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(54) Title: PHARMACEUTICAL COMPOSITIONS COMPRISING POLYNUCLEOTIDES ENCODING A RAF PROTEIN

(57) Abstract: Disclosed are pharmaceutical compositions comprising polynucleotides encoding a Raf protein, vectors, host cells, polypeptides encoded by said polynucleotides as well as agonists or antagonists thereof. Furthermore, described are uses of such pharmaceutical compositions for preventing or treating pathological conditions in which endothelial cells are involved or affected. Finally, methods for screening compounds acting as agonists or antagonists as well as diagnostic compositions and methods are disclosed.

PHARMACEUTICAL COMPOSITIONS COMPRISING POLYNUCLEOTIDES ENCODING A RAF PROTEIN

The present invention relates to pharmaceutical compositions comprising polynucleotides encoding a Raf protein, vectors, host cells, polypeptides encoded by said polynucleotides and agonists or antagonists thereof. The invention furthermore relates to uses of such pharmaceutical compositions for preventing or treating pathological conditions in which endothelial cells are involved or affected. Furthermore, the invention relates to methods for screening compounds acting as agonists or antagonists as well as to diagnostic compositions and methods.

Endothelial cells form the inner wall surface of blood vessels. The presence of endothelium in all tissues, and its central role in numerous vital functions such as tissue survival and blood clotting, places the vascular system at the core of many pathological conditions that affect the human population. Angiogenesis, the formation of new blood vessels, is essential for embryonic development, subsequent growth and tissue repair. This process is also essential in several pathological conditions, such as neoplasias. Generally, endothelial cells play a critical role in diseases such as cancer, stroke, cardiac infarction, circulatory problems, tissue injury, diabetic retinopathy, psoriasis, inflammation and atherosclerosis.

However, to date the molecular mechanisms that take place in the course of pathological conditions where endothelial cells are involved or affected are still poorly understood. A more detailed knowledge of the signal transduction and gene regulation pathways within endothelial cells would be promising for identifying or generating pharmacologically valuable compounds that may specifically influence disease states localized in endothelial cells. In this regard, it would be favorable to have access to molecules which are capable of effectively down or upregulating the activity of genes that are known to be involved in pathological conditions, as for example those outlined above.

Thus, the technical problem underlying the present invention is to provide means and methods for preventing or treating pathological conditions in which endothelial cells are involved or affected.
This technical problem is solved by the provision of the embodiments as characterized in the claims.

Accordingly, the present invention relates to pharmaceutical compositions comprising a compound selected from the group consisting of

(i) polynucleotides encoding a Raf protein selected from the group consisting of
  (a) polynucleotides encoding a polypeptide comprising the amino acid sequence of any one of SEQ ID NOs: 2, 4 and 6;
  (b) polynucleotides comprising the coding region of the nucleotide sequence of any one of SEQ ID NOs: 1, 3 and 5;
  (c) polynucleotides encoding a fragment of a polypeptide encoded by a polynucleotide of (a) or (b); and
  (d) polynucleotides the complementary strand of which hybridizes with a polynucleotide of any one of (a) to (c) and encode a polynucleotide having Raf activity;
(ii) vectors comprising and capable of expressing said polynucleotide;
(iii) host cells genetically engineered with said polynucleotide or said vector;
(iv) polypeptides encoded by said polynucleotide; and
(v) agonists of a polypeptide encoded by said polynucleotide;

and optionally a pharmaceutically acceptable carrier.

The present invention relates in a further embodiment to pharmaceutical compositions comprising a compound which is an antagonist of the polypeptide encoded by a polynucleotide as defined above and optionally a pharmaceutically acceptable carrier. The term "nucleic acid molecule" and "polynucleotide" are used interchangeably herein. The term "and/or" wherever used herein includes the meaning of "and", "or" and "any other combination of the elements connected by said term".

The invention, thus, relates in one aspect to pharmaceutical compositions that are based on a protein that belongs to the Raf protein kinase family and to polynucleotide encoding such proteins as identified above. The gene family of Raf protein kinases comprises the genes encoding the protein kinases A-Raf, B-Raf and C-Raf, from which the amino acid sequences of the human representatives is shown in the SEQ ID NOs: 2, 4 and 6, respectively. Corresponding cDNA sequences encoding these human A-Raf, B-Raf and C-Raf proteins are shown under SEQ ID NOs: 1, 3 and 5, respectively. The Raf proteins are protein kinases that are involved in numerous signal transduction cascades in eukaryotic cells. As a preferred embodiment, the present invention relates to pharmaceutical compositions, wherein the above mentioned Raf protein is B-Raf.

The present invention is based on the surprising finding that in a mouse embryonic endothelial progenitor cell (eEPC) line in which the gene encoding B-Raf is inactivated, in the following also referred to as B-Raf KO (knock out) or null cells, the expression profile of a large set of genes is altered compared to the corresponding wild-type (wt) cells. In the experiments which are described in detail in the appended Example 1, it could be shown that of the approximately 1500 genes so far studied about 5% to 10% are up or down regulated on the transcriptional level in the B-Raf KO cells (see Table 1). If one extrapolates from these results to the ten-thousands of genes that are normally expressed in wild-type embryonic endothelial cells, it is reasonable to anticipate that several hundred to several thousand cellular genes show an altered gene expression in B-Raf KO cells and are therefore influenced by signaling pathways under control of B-Raf.

In subsequent experiments, these results obtained with B-Raf null cells were confirmed for the situation in vivo by comparing gene expression profiles between wt and B-Raf KO embryos (Example 2). The results show that genes, whose expression changed in the B-Raf null cells, were affected during embryonic vascular development, i.e. they were also deregulated.

Further evidence that B-Raf is essential for protein regulation in endothelial cells is provided by the fact that cAMP can induce the activation of the B-Raf mediated MAP kinase pathway in eEPCs as shown in Example 3. These results suggest that the effects of a number of divers extracellular signals that modulate intracellular cAMP

levels in endothelial cells, and thus lead to MAPK pathway activation, can in turn be modulated by agents that affect B-Raf activity.

In addition, the electron microscopy data shown in Example 4 and Figure 4, reveal B-Raf's importance for the proper establishment of the endothelial/peri-endothelial environment. This and the similarity to the angiogenin 1 (Ang1) KO mice phenotype observed, which points at a position of B-Raf downstream from tie-2 in the signaling cascade, substantiates the role of B-Raf in angiogenesis, wound healing and endothelial cell migration.

These data might explain the fact that the B-Raf KO genotype is lethal for homozygous knock out mice already in the mid-gestation phase and that in such mice endothelial cells are extensively affected by apoptosis (Wojnowski, Nature Genetics 16 (1997), 293-297).

This central role of B-Raf in the gene regulation of endothelial cells was unexpected because B-Raf protein is not endothelial-specific but is expressed in many cell types (Bernier, J. Biol. Chem. 270 (1995), 23381-23389) and, in particular, rather extensively in neuronal tissues (Jaiswal et al., Mol. Cell. Biol. 14 (1994), 6944-6953).

The consequences of the B-Raf pathway impairment in endothelial cells were previously not known. The above-mentioned results demonstrate for the first time that gene expression of a large number of genes is altered and that the affected genes belong to molecular pathways with crucial roles in endothelial cell functions such as angiogenesis, thrombosis, atherosclerosis and inflammation.

These data lead to two major conclusions: (a) the results obtained in B-Raf null cells are not an artifact of the cell isolation procedure because of their reproducibility in B-Raf KO embryos, and (b) the documented changes in gene expression profiles are a direct consequence of the lack of B-Raf protein in endothelial cells. Consequently, B-Raf has a central regulatory role in vascular system diseases that affect endothelial cells.

It is therefore conceivable to use pharmaceutical compositions suitable for modulation of B-Raf activity in therapeutic approaches for pathological conditions in which endothelial cells are involved or affected, such as tumor-induced angiogenesis, diabetic retinopathy, wound healing, psoriasis, inflammation, stroke, myocardial infarction, atherosclerosis and arterial restenosis.

The therapeutic uses conceivable for compounds modulating B-Raf activity in endothelial cells are also envisaged for A-Raf and C-Raf because biochemical analyses have shown that the distinct functions of the three types of kinases overlap to a high degree (Pritchard, Mol. Cell. Biol. 15 (1995), 6430-6442). Moreover, genetic and phenotypic analysis in single gene KO mice and in double B-Raf and C-Raf KO mice show that the functions for example of B-Raf and C-Raf are overlapping but not redundant (Wojnowski, Mechanisms of Development 91 (2000), 97-104).

In one embodiment, the pharmaceutical compositions of the invention comprise a compound which is a polynucleotide encoding a Raf protein.

The term "Raf protein" refers to a protein encoded by a member of the Raf protein kinase gene family. In particular, this gene family encodes three different types of protein kinases, A-Raf, B-Raf and C-Raf. As a common feature, these proteins are part of various signal transduction pathways. Structure, activity and functions of the members of the Raf kinase gene family are described in detail in the prior art as for instance reviewed in Morrison and Cutler (Current Opinion in Cell Biology 9 (1997), 174-179). A common feature of the Raf family of protein kinases is that they all share three highly conserved regions called CR1, CR2 and CR3. CR1 is rich in cysteine residues while CR2 contains many serines and theorines. The CR3 domain contains the kinase activity. In accordance with the sequence comparisons described in Sithanandam (Oncogene 5 (1999), 1775-1780), CR1 and CR3 are highly conserved among all three types of protein kinases, preferably having a homology, that is to say amino acid sequence identity of more than 90%, more preferably of more than 93%. CR2, in contrast thereto, is highly conserved only between A-Raf and C-Raf, while it is less conserved in B-Raf, having a homology of less than 60%, preferably of less than 50% compared to CR2 of A-Raf and C-Raf. The naturally occurring Raf proteins feature considerable size differences. On average, B-Raf proteins have a molecular weight of about 90 kDa and A-Raf and C-Raf of about 70 kDa. Correspondingly, the amino acid sequence of A-Raf and C-Raf is at least 100 residues shorter than that of B-Raf. Examples of proteins that are capable of activating Raf proteins include ras and Protein Kinase A (PKA). Another kinase, Rap1, is capable of phosphorylating B-Raf while it does not phosphorylate C-Raf. The kinase activity of Raf proteins leads to phosphorylation of specific substrate proteins, thereby leading to an activation or

inactivation, preferably to an activation, of the substrate protein. Examples of pathways that Rafs are involved in are the MAP kinase pathway and the MEK/ERK pathway (Avruch, Trends Biochem. Sci. 19 (1994), 279-283). Specifically B-Raf is capable of phosphorylating the proteins MEK 1 and 2 (Heidecker, Mol. Cell. Biol. 10 (1990), 2503-2512; Reuter, J. Biol. Chem. 270 (1995), 7644-7655). Suitable assays for determining Raf protein activity are described in the literature, for instance in Reuter (J. Biol. Chem. 270 (1995), 7644-7655), Jaiswal (Mol. Cell. Biol. 14 (1994), 6944-6953) and Voltek (Cell 17 (1993), 205-214; Wang, Cell 87 (1996), 629-638). A specific example of a kinase assay to monitor Raf kinase activity is as follows: Raf kinases are purified from eukaryotic cells following immunoprecipitation with Anti-Raf antibodies. The collected complexes are washed in salt/HEPES buffer and then incubated with baculovirus-produced MEK protein in the presence of radioactive gamma ATP. Following incubation for 30 min at 30°C, the reaction products (phosphorylated MEK) are resolved in 10% polyacrylamide gels, visualized by autoradiography and counted using a Phosphoimager. Suitable assays to distinguish A-, B- and C-Raf activity may use differences regarding potency in activating MEK. For instance, according to an in vitro assay described by Pritchard (Mol. Cell. Biol. 15 (1995), 6430-6442), B-Raf phosphorylating activity is about 10 times stronger than that of C-Raf and about 500 times stronger than that of A-Raf. According to another approach, specific antibodies against A-, B- and C-Raf can be applied to monitor their phosphorylation status (Reuter, J. Biol. Chem. 270 (1995), 7644-7655).

Preferably, the polynucleotide encodes a Raf protein comprising the amino acid sequence of the human A-Raf, B-Raf or C-Raf protein, i.e. the sequences shown in SEQ ID NOs: 2, 4 and 6, respectively. Such polypeptides are preferably encoded by the polynucleotides comprising the coding region of the nucleotide sequence shown in SEQ ID NOs: 1, 3 and 5, respectively, or are retrievable from the GeneBank/EMBL data base entries having the accession Nos. X04790, M95712 and X03484, respectively. As a preferred embodiment, said polynucleotides encode a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 or comprises the coding region of the nucleotide sequence of SEQ ID NO: 3.

The polynucleotide contained in the pharmaceutical composition of the invention may as well encode a fragment or an epitope-bearing portion of a polypeptide as

described above. Preferably, such fragments have the activity of a Raf protein. In particular, the length of such a fragment is not below 10 amino acid residues, preferably not below 20, more preferably not below 50 and most preferably not below 100 amino acid residues.

In another embodiment the polynucleotide contained in the pharmaceutical composition is a polynucleotide the complementary strand of which hybridizes with one of the above-described polynucleotides and which encodes a Raf protein.
Also included in this context are polynucleotides which encode a protein, which has a homology, that is to say a sequence identity, of at least 30%, preferably of at least 40%, more preferably of at least 50%, even more preferably of at least 60% and particularly preferred of at least 70%, especially preferred of at least 80% and even more preferred of at least 90% to the entire amino acid sequence as indicated in SEQ ID NO: 2, 4, or 6 the protein being a Raf protein.
Hybridizing polynucleotides also encompass polynucleotides which encode a Raf protein and the nucleotide sequences of which have a homology, that is to say a sequence identity, of at least 40%, preferably of at least 50%, more preferably of at least 60%, even more preferably of at least 70%, in particular of at least 80%, especially preferred of at least 90%, in particular of at least 95% and even more preferred of at least 99% when compared to the coding region of the sequence shown in SEQ ID NO:1, 3 or 5.

In the context of the present invention the term "hybridization" means hybridization under conventional hybridization conditions, preferably under stringent conditions, as for instance described in Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. In an especially preferred embodiment the term "hybridization" means that hybridization occurs under the following conditions:

Hybridization buffer: 2 x SSC; 10 x Denhardt solution (Fikoll 400 + PEG + BSA; ratio 1:1:1); 0.1% SDS; 5 mM EDTA; 50 mM Na_2HPO_4;

	250 µg/ml of herring sperm DNA; 50 µg/ml of tRNA;
	or
	0.25 M of sodium phosphate buffer, pH 7.2;
	1 mM EDTA
	7% SDS
Hybridization temperature T	= 60°C
Washing buffer:	2 x SSC; 0.1% SDS
Washing temperature T	= 60°C.

Polynucleotides which hybridize with the polynucleotides as specified above can, in principle, encode a Raf protein from any organism expressing such proteins or can encode modified versions thereof.

Polynucleotides which hybridize with the polynucleotides as specified above can for instance be isolated from genomic libraries or cDNA libraries of bacteria, fungi, plants or animals. Preferably, such molecules are from animal origin, e.g., from vertebrate or evertebrate animals, more preferably from mammal origin and particularly preferred from human origin. It is furthermore preferred that the polynucleotide used in the pharmaceutical composition of the invention is isolated from the same species as the species to which the pharmaceutical composition is to be administered. In this regard, the species may be any one to which administration of the pharmaceutical composition is useful, for example to vertebrates, preferably to mammals. Preferred mammals are rodents (e.g. mouse or rat), working animals (e.g. cow, horse, pig), other domestic animals (e.g. dog, cat), animals commonly used in animal experiments (in addition to other already mentioned species, e.g., rabbit, monkey), and most preferred are humans. As an alternative to isolate said polynucleotides from natural sources, they can be prepared by genetic engineering or chemical synthesis.

Polynucleotides useful for preparing the pharmaceutical composition of the invention may be identified and isolated by using the above-described polynucleotides or parts of these polynucleotides or reverse complements of these polynucleotides, for instance by hybridization according to standard methods (see for instance Sambrook et al., 1989, Molecular Cloning. A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Nucleic acid molecules comprising the same or substantially the same nucleotide sequence as indicated in SEQ ID NO: 1, 3 or 5 or fragments thereof can, for instance, be used as hybridization probes. Such fragments used as hybridization probes can also be synthetic fragments which are prepared by usual synthesis techniques.

The polynucleotides hybridizing with the above-described polynucleotides also comprise fragments, derivatives and allelic variants of said above-described polynucleotides encoding a Raf protein. Herein, fragments are understood to mean parts of the polynucleotides which are long enough to encode the described protein, preferably showing the biological activity of a Raf protein described above, e.g. being capable of activating MEK protein and/or the MEK/ERK signaling cascade. In this context, the term derivative means that the sequences of these molecules differ from the sequences of the above-described polynucleotides in one or more positions and show a high degree of homology to these sequences. In this context, homology means a sequence identity of at least 40%, in particular an identity of at least 60%, preferably of more than 65%, even more preferably of at least 70%, in particular of at least 80%, more preferably of at least 90% and particularly preferred of more than 95%. Deviations from the above-described polynucleotides may have been produced, e.g., by deletion, substitution, insertion and/or recombination.

Preferably, the degree of homology is determined by comparing the respective sequence with the nucleotide sequence of the coding region of SEQ ID NO: 1, 3 or 5. When the sequences which are compared do not have the same length, the degree of homology preferably refers to the percentage of nucleotide residues in the shorter sequence which are identical to nucleotide residues in the longer sequence. The degree of homology can be determined conventionally using known computer programs such as the DNASTAR program with the ClustalW analysis. This program can be obtained from DNASTAR, Inc., 1228 South Park Street, Madison, WI 53715 or from DNASTAR, Ltd., Abacus House, West Ealing, London W13 0AS UK (support@dnastar.com) and is accessible at the server of the EMBL outstation.

When using the Clustal analysis method to determine whether a particular sequence is, for instance, 80% identical to a reference sequence the settings are preferably as follows: Matrix: blosum 30; Open gap penalty: 10.0; Extend gap penalty: 0.05; Delay

divergent: 40; Gap separation distance: 8 for comparisons of amino acid sequences. For nucleotide sequence comparisons, the Extend gap penalty is preferably set to 5.0.

Furthermore, homology means preferably that the encoded protein displays a sequence identity of at least 30%, more preferably of at least 40%, even more preferably of at least 50%, in particular of at least 60%, particularly preferred of at least 70%, especially preferred of at least 80% and even more preferred of at least 90% to the amino acid sequence depicted under SEQ ID NO: 2, 4 or 6.

Preferably, the degree of homology of the hybridizing polynucleotides is calculated over the complete length of its coding sequence. It is furthermore preferred that such a hybridizing nucleic acid molecule, and in particular the coding sequence comprised therein, has a length of at least 200 nucleotides, preferably at least 400 nucleotides, more preferably of at least 600 nucleotides, even more preferably of at least 800 nucleotides, particularly preferred of at least 1000 nucleotides and most preferably of at least 1500 nucleotides.

Preferably, sequences hybridizing to a polynucleotide as described above comprise a region of homology of at least 90%, preferably of at least 93%, more preferably of at least 95%, still more preferably of at least 98% and particularly preferred of at least 99% identity to said polynucleotide, wherein this region of homology has a length of at least 300 nucleotides, more preferably of at least 500 nucleotides, even more preferably of at least 750 nucleotides, particularly preferred of at least 1000 nucleotides and most preferably of at least 1500 nucleotides.

Homology, moreover, means that there is a functional and/or structural equivalence between the corresponding polynucleotides or proteins encoded thereby. Polynucleotides which are homologous to the above-described molecules and represent derivatives of these molecules are normally variations of these molecules which represent modifications having the same biological function. They may be either naturally occurring variations, for instance sequences from other allelic variants, varieties, species, etc., or mutations, and said mutations may have formed naturally or may have been produced by deliberate mutagenesis. Furthermore, the variations may be synthetically produced sequences. The allelic variants may be naturally occurring variants or synthetically produced variants or variants produced by recombinant DNA techniques.

The proteins encoded by the different variants of the above-described polynucleotides possess certain characteristics they have in common. These include for instance biological activity, molecular weight, immunological reactivity, conformation, etc., and physical properties, such as for instance the migration behavior in gel electrophoreses, chromatographic behavior, sedimentation coefficients, solubility, spectroscopic properties, stability, pH optimum, temperature optimum etc.
The biological activity of the Raf protein, in particular the capacity to phosphorylate MEK or can be tested as described above, e.g. by an assay as described in Reuter (J. Biol. Chem. 270 (1995), 7644-7655).

The polynucleotide contained in the pharmaceutical composition of the invention may also comprise in addition to the coding sequence for a Raf protein, as for example the polypeptide having the amino acid sequence of SEQ ID NO: 2, 4 or 6 or a polypeptide homologous thereto, further coding or non-coding nucleotide sequences. Thus, for instance, the polynucleotide may encode one of the above-described polypeptides which is fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. The marker sequence may for example be a hexa-histidine peptide, such as the tag contained in a pQE vector (Qiagen, Inc.) which provides for convenient purification of the fusion protein. Another suitable marker sequence may be the HA tag which corresponds to an epitope derived from influenza hemagglutinin protein (Wilson, Cell 37 (1984), 767). A further example is the coding sequence of glutathione-S-transferase (GST) which, apart from providing a purification tag, enhances protein stability, for instance, in bacterial expression systems.

The polynucleotide contained in the pharmaceutical composition of the invention can be any type of polynucleotide, e.g. DNA molecules or RNA molecules or combinations thereof. These polynucleotides can be obtained for instance from natural sources or may be produced synthetically or by recombinant techniques, such as PCR. Such polynucleotides may comprise any modification thereof that is known in the state of the art (see, e.g., US 5525711, US 4711955, US 5792608 or EP 302175 for examples of modifications). Such polynucleotides may be single- or

double-stranded, linear or circular, without any size limitation. For instance, the polynucleotide(s) may be genomic DNA, cDNA, mRNA etc.

The polynucleotides described hereinabove allow to prepare host cells as well as to produce recombinantly proteins having Raf activity of high purity and/or in sufficient quantities for preparing pharmaceutical compositions comprising any of these compounds.

Another compound that may be comprised in a pharmaceutical composition of the invention is a vector comprising and capable of expressing a polynucleotide as described above. Such a vector can be an expression vector and/or a gene delivery vector. Expression vectors are in this context meant for use in ex vivo gene therapy techniques, i.e. suitable host cells are transfected outside the body and then administered to the subject. Gene delivery vectors are referred to herein as vectors suited for in vivo gene therapeutic applications, i.e. the vector is directly administered to the subject, either systemically or locally. The vector referred to herein may only consist of nucleic acid or may be complexed with additional compounds that enhance, for instance, transfer into the target cell, targeting, stability and/or bioavailability, e.g. in the circulatory system. Examples of such additional compounds are lipidic substances, polycations, membrane-disruptive peptides or other compounds, antibodies or fragments thereof or receptor-binding molecules specifically recognizing the target cell, etc. Expression or gene delivery vectors may preferably be derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses or bovine papilloma virus, and may be used for delivery into a targeted cell population, e.g. into endothelial cells. Methods which are well known to those skilled in the art can be used to construct recombinant expression or gene delivery vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the vectors can be reconstituted into liposomes for delivery to target cells. The vectors containing the above-described polynucleotides can be transferred into a host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium

chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts; see Sambrook, loc. cit.

The vectors referred to herein may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions. Preferably, the polynucleotide comprised in the vector is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic cells. Expression of said polynucleotide comprises transcription of the polynucleotide into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They usually comprise regulatory sequences ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript, and/or an intron further enhancing expression of said polynucleotide. Additional regulatory elements may include transcriptional as well as translational enhancers, and/or naturally-associated or heterologous promoter regions. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the PL, lac, trp or tac promoter in E. coli, and examples for regulatory elements permitting expression in eukaryotic host cells are the AOX1 or GAL1 promoter in yeast or the CMV-, SV40- , RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian or other animal cells. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. Furthermore, depending on the expression system used leader sequences capable of directing the polypeptide to a cellular compartment or secreting it into the extracellular space may be added to the coding sequence of the polynucleotide and are well known in the art. The leader sequence(s) is (are) assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of the translated protein, or a portion thereof, into the periplasmic space or extracellular space. Optionally, the heterologous sequence can encode a fusion protein including an C- or N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product. In this context, suitable expression vectors are known in the art such as

Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), or pSPORT1 (GIBCO BRL).

Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used.

As mentioned above, the vector described herein is for use in gene therapy, which is based on introducing therapeutic genes into cells by ex-vivo or in-vivo techniques. Suitable vectors and methods for ex-vivo or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., Giordano, Nature Medicine 2 (1996), 534-539; Schaper, Circ. Res. 79 (1996), 911-919; Anderson, Science 256 (1992), 808-813; Isner, Lancet 348 (1996), 370-374; Muhlhauser, Circ. Res. 77 (1995), 1077-1086; Wang, Nature Medicine 2 (1996), 714-716; WO 94/29469; WO 97/00957 or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640, and references cited therein. The vectors comprised in the pharmaceutical composition of the invention may be designed for direct introduction or for introduction via liposomes or viral vectors (e.g. adenoviral, retroviral) into the cell. Preferably, said cell is a germ line cell, embryonic cell or egg cell or a cell derived therefrom, most preferably said cell is a stem cell.

In a further embodiment, the pharmaceutical compositions of the invention comprise host cells genetically engineered with the above-described polynucleotides or vectors.

Said host cell may in principle be a prokaryotic or eukaryotic cell. Said polynucleotide or vector which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained extrachromosomally. The term "prokaryotic" is meant to include all bacteria which can be transformed or transfected with said polynucleotides or vectors. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, E. coli, S. typhimurium, Serratia marcescens and Bacillus subtilis. The term "eukaryotic" is meant to include fungal higher plant, insect and preferably mammalian cells. Preferred fungal cells are yeast cells, for example, those of the genus Saccharomyces, in particular those of the species S. cerevisiae. Among the mammalian cells human cells are most preferred.

The transformation or transfection of the host cell with a polynucleotide or vector as described above can be carried out by standard methods, as for instance described

in Sambrook et al., (Molecular Cloning: A Laboratory Manual, 2nd edition (1989) Cold Spring Harbor Press, New York; Methods in Yeast Genetics, A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, 1990).

The herein described host cells may be of therapeutic value in ex-vivo gene therapy, in particular by targeting them to the endothelium using a suitable targeting technique. Advantageously, such host cells are endothelial cells or progenitor cells thereof. For example, transplantation of genetically modified cells can be used for therapeutic purposes by providing endothelial cells with desired traits such as resistance to angiogenic signals or thromboresistence.

Furthermore, the pharmaceutical composition of the invention may comprise a polypeptide encoded by the above-described polynucleotide.

This polypeptide may, e.g., be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture).

Provision of the polypeptide via recombinant expression of one of the above-described polynucleotides is preferred. An overview of different expression systems is for instance contained in Methods in Enzymology 153 (1987), 385-516, in Bitter et al. (Methods in Enzymology 153 (1987), 516-544) and in Sawers et al. (Applied Microbiology and Biotechnology 46 (1996), 1-9), Billman-Jacobe (Current Opinion in Biotechnology 7 (1996), 500-4), Hockney (Trends in Biotechnology 12 (1994), 456-463), Griffiths et al., (Methods in Molecular Biology 75 (1997), 427-440). An overview of yeast expression systems is for instance given by Hensing et al. (Antonie van Leuwenhoek 67 (1995), 261-279), Bussineau et al. (Developments in Biological Standardization 83 (1994), 13-19), Gellissen et al. (Antonie van Leuwenhoek 62 (1992), 79-93, Fleer (Current Opinion in Biotechnology 3 (1992), 486-496), Vedvick (Current Opinion in Biotechnology 2 (1991), 742-745) and Buckholz (Bio/Technology 9 (1991), 1067-1072).

Expression vectors have been widely described in the literature. As a rule, they contain not only a selection marker gene and a replication-origin ensuring replication in the host selected, but also a bacterial or viral promoter, and in most cases a termination signal for transcription. Between the promoter and the termination signal

there is in general at least one restriction site or a polylinker which enables the insertion of a coding DNA sequence. The DNA sequence naturally controlling the transcription of the corresponding gene can be used as the promoter sequence, if it is active in the selected host organism. However, this sequence can also be exchanged for other promoter sequences. It is possible to use promoters ensuring constitutive expression of the gene and inducible promoters which permit a deliberate control of the expression of the gene. Bacterial and viral promoter sequences possessing these properties are described in detail in the literature. Regulatory sequences for the expression in microorganisms (for instance *E. coli, S. cerevisiae)* are sufficiently described in the literature. Promoters permitting a particularly high expression of a downstream sequence are for instance the T7 promoter (Studier et al., Methods in Enzymology 185 (1990), 60-89), lacUV5, trp, trp-lacUV5 (DeBoer et al., in Rodriguez and Chamberlin (Eds), Promoters, Structure and Function; Praeger, New York, (1982), 462-481; DeBoer et al., Proc. Natl. Acad. Sci. USA (1983), 21-25), lp1, rac (Boros et al., Gene 42 (1986), 97-100). Inducible promoters are preferably used for the synthesis of proteins. These promoters often lead to higher protein yields than do constitutive promoters. In order to obtain an optimum amount of protein, a two-stage process is often used. First, the host cells are cultured under optimum conditions up to a relatively high cell density. In the second step, transcription is induced depending on the type of promoter used. In this regard, a tac promoter is particularly suitable which can be induced by lactose or IPTG (=isopropyl-ß-D-thiogalactopyranoside) (deBoer et al., Proc. Natl. Acad. Sci. USA 80 (1983), 21-25). Termination signals for transcription are also described in the literature.

Transformation or transfection of suitable host cells can be carried out according to one of the methods mentioned above. The host cell is cultured in nutrient media meeting the requirements of the particular host cell used, in particular in respect of the pH value, temperature, salt concentration, aeration, antibiotics, vitamins, trace elements etc. The polypeptide having Raf activity can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography.

Protein refolding steps can be used, as necessary, in completing configuration of the protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

Depending upon the host employed in a recombinant production procedure, the polypeptide may be glycosylated or may be non-glycosylated. The polypeptide may also include an initial methionine amino acid residue. The polypeptide may be further modified to contain additional chemical moieties not normally part of a naturally occurring protein. Those derivatized moieties may, e.g., improve the stability, solubility, the biological half life or absorption of the polypeptide. The moieties may also reduce or eliminate any undesirable side effects of the polypeptide and the like. An overview for these moieties can be found, e.g., in Remington's Pharmaceutical Sciences (18^{th} ed., Mack Publishing Co., Easton, PA (1990)). Polyethylene glycol (PEG) is an example for such a chemical moiety which has been used for the preparation of therapeutic proteins. The attachment of PEG to proteins has been shown to protect them against proteolysis (Sada et al., J. Fermentation Bioengineering 71 (1991), 137-139). Various methods are available for the attachment of certain PEG moieties to proteins (for review see: Abuchowski et al., in "Enzymes as Drugs"; Holcerberg and Roberts, eds. (1981), 367-383). Generally, PEG molecules are connected to the protein via a reactive group found on the protein. Amino groups, e.g. on lysines or the amino terminus of the protein are convenient for this attachment among others.

The polypeptides comprised in the pharmaceutical compositions of the present invention can comprise a further domain, said domain being linked by covalent or non-covalent bonds. The linkage can be based on genetic fusion according to the methods known in the art and described above or can be performed by, e.g., chemical cross-linking as described in, e.g., WO 94/04686. The additional domain present in the fusion protein comprising the polypeptide employed in accordance with the invention may preferably be linked by a flexible linker, advantageously a polypeptide linker, wherein said polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of said further domain and the N-terminal end of the polypeptide or vice versa. The above described fusion protein may further comprise a cleavable linker or cleavage site for proteinases.

Furthermore, said further domain may be of a predefined specificity or function. In this context, it is understood that the polypeptides present in the pharmaceutical composition according to the invention may be further modified by conventional methods known in the art. This allows for the construction of fusion proteins comprising the polypeptide of the invention and other functional amino acid sequences, e.g., nuclear localization signals, transactivating domains, DNA-binding domains, hormone-binding domains, protein tags (GST, GFP, h-myc peptide, FLAG, HA peptide) which may be derived from heterologous proteins. Thus, administration of the composition of the invention can utilize unlabeled as well as labeled polypeptides.

The pharmaceutical compositions of the invention may also comprise compounds that have an agonistic effect on the polypeptide having Raf activity as described above.

Preferably, said agonist compound can be identified by a method for screening such compounds comprising the steps of

(a) measuring the activity of a polypeptide as described above in the presence and in the absence of the compound to be tested; and

(b) identifying a compound acting as an agonist if the activity measured in step (a) is higher in the presence of the compound than in the absence of the compound.

Screening methods for identifying compounds that influence the activity of a given protein are well known in the art and can be taken from the literature. Preferably, such a screening is carried out in a high-throughput fashion with a degree of automation as high as possible. Candidate compounds may for instance be provided from libraries of chemical or biological substances that are routinely taken for such approaches and are known in the art. The activity assayed in step (a) is preferably the kinase activity of the Raf protein. The kinase activity may be measured as described above. Further activity assays are for instance described in Reuter (J. Biol. Chem. 270 (1995), 7644-7655), Jaiswal (Mol. Cell. Biol. 14 (1994), 6944-6953), Voltek (Cell 74 (1993), 205-214) and Wang (Cell 87 (1996), 629-638). For instance, such an activity assay can be carried out as described above, i.e. using

recombinantly produced MEK in the presence of radioactively labeled gamma ATP. One could furthermore use an apoptosis assay (e.g. the TUNEL ASSAY) or one could do RNA analysis for changes in gene expression based on the genes identified in the context of the present invention (see Table 1).

The present invention furthermore relates to pharmaceutical compositions comprising a compound which is an antagonist of the above-described polypeptide having Raf kinase activity.

Potential Raf protein antagonists include antibodies of fragments thereof or oligonucleotides which bind to the polypeptide and effectively reduce Raf kinase activity.

Antibodies useful as antagonists can be monoclonal or polyclonal and can be prepared according to methods well known in the art. The term "antibody" also comprises fragments of an antibody which still retain the binding specificity.

The polypeptide as described above, its fragments or other derivatives thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. In particular, also included are chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of a Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies directed against a polypeptide as described above can be obtained, e.g., by direct injection of the protein into an animal or by administering the polypeptide to an animal, preferably a non-human animal. The antibody so obtained will then bind the protein itself. In this manner, even a sequence encoding only a fragment of the protein can be used to generate antibodies binding the whole native polypeptide.

Such antibodies can then, e.g., be used to isolate the protein from tissue expressing that polypeptide or to detect it in a probe. For the preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples for such techniques include the hybridoma technique (Köhler and Milstein, Nature 256 (1975), 495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today 4 (1983), 72) and the EBV-hybridoma technique to produce human monoclonal antibodies

(Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. (1985), 77-96). Techniques describing the production of single chain antibodies (e.g., U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptides as described above. Furthermore, transgenic mice may be used to express humanized antibodies directed against said immunogenic polypeptides.

Alternatively, a potential antagonist may be a mutant form of the polypeptide, preferably a dominant mutant form, which is for example inactive with regard to biological activity, e.g. to kinase activity. Such a mutant form could, for instance, bind to the natural Raf substrate protein but without activating it through phosphorylation. Alternatively or in addition, the mutant form could still have a phosphorylation site whereby phosphorylation of this site does not result in Raf activation. Such inactivated mutant forms of Raf proteins would be suited to block the natural Raf-mediated signal transduction pathway. Since protein structures of A-, B-, as well as of C-Raf proteins are well known in the art, the person skilled in the art is capable of designing such mutant forms from the corresponding wild-type Raf protein by way of amino acid deletion(s), substitution(s) and/or addition(s) in the amino acid sequence of the protein.

Another class of potential antagonist compounds comprises nucleic acid molecules that are capable of reducing Raf protein activity in a cell by way of intervening into gene expression of said protein, such as for example antisense, sense, ribozyme or co-suppression constructs. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, whereby the inhibitory effect is based on specific binding of a nucleic acid molecule to DNA or RNA. For example, the 5' coding portion of a nucleic acid molecule encoding a Raf protein to be inhibited can be used to design an antisense RNA oligonucleotide, e.g., of from about 10 to 40 nucleotides in length. The antisense DNA or RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of said mRNA and/or leads to destabilization of the mRNA molecule (Okano, J. Neurochem. 56 (1991), 560; Oligodeoxynucleotides as antisense inhibitors of gene expression, CRC Press, Boca Raton, FL, USA (1988)). For applying a triple-helix approach, a DNA

oligonucleotide can be designed to be complementary to a region of the gene encoding a Raf protein to be inhibited according to the principles laid down in the prior art (see for example Lee, Nucl. Acids Res. 6 (1979), 3073; Cooney, Science 241 (1988), 456; and Dervan, Science 251 (1991), 1360). Such a triple-helix forming oligonucleotide can then be used to prevent transcription of the specific gene. The oligonucleotides described above can also be delivered to target cells via a gene delivery vector as described above in order to express such molecules in vivo to inhibit gene expression of the respective Raf protein.

Examples for antisense molecules are oligonucleotides specifically hybridizing to a polynucleotide encoding a polypeptide having Raf activity. Such oligonucleotides have a length of preferably at least 10, in particular at least 15, and particularly preferably of at least 50 nucleotides. They are characterized in that they specifically hybridize to said polynucleotide, that is to say that they do not or only to a very minor extent hybridize to other nucleic acid sequences.

Likewise, RNA molecules with ribozyme activity which specifically cleave transcripts of a gene encoding a Raf protein or DNA molecules encoding such RNAs can be used. Ribozymes are catalytically active RNA molecules capable of cleaving RNA molecules and specific target sequences. By means of recombinant DNA techniques it is possible to alter the specificity of ribozymes. There are various classes of ribozymes. For practical applications aiming at the specific cleavage of the transcript of a certain gene, use is preferably made of representatives of two different groups of ribozymes. The first group is made up of ribozymes which belong to the group I intron ribozyme type. The second group consists of ribozymes which as a characteristic structural feature exhibit the so-called "hammerhead" motif. The specific recognition of the target RNA molecule may be modified by altering the sequences flanking this motif. By base pairing with sequences in the target molecule these sequences determine the position at which the catalytic reaction and therefore the cleavage of the target molecule takes place. Since the sequence requirements for an efficient cleavage are low, it is in principle possible to develop specific ribozymes for practically each desired RNA molecule.

In order to produce DNA molecules encoding a ribozyme which specifically cleaves transcripts of a gene encoding a Raf protein, for example a DNA sequence encoding a

catalytic domain of a ribozyme is bilaterally linked with DNA sequences which are homologous to sequences encoding the target protein. Sequences encoding the catalytic domain may for example be the catalytic domain of the satellite DNA of the SCMo virus (Davies, Virology 177 (1990), 216-224 and Steinecke, EMBO J. 11 (1992), 1525-1530) or that of the satellite DNA of the TobR virus (Haseloff and Gerlach, Nature 334 (1988), 585-591). The DNA sequences flanking the catalytic domain are preferably derived from the above-described polynucleotides contained in a pharmaceutical composition of the invention. The expression of ribozymes in order to decrease the activity in certain proteins in cells is also known to the person skilled in the art and is, for example, described in EP-B1 0 321 201.

Further potential antagonist compounds to Raf proteins include small molecules which bind to and occupy the active site of the protein thereby making the catalytic site inaccessible to substrate such that normal biological activity is prevented. Examples of small molecules include small peptides or peptide-like molecules.

Preferably, said compounds having an antagonistic effect on the polypeptide having Raf activity can be identified by a method for screening such compounds comprising the steps of

(a) measuring the activity of said polypeptide in the presence and in the absence of the compound to be tested; and

(b) identifying a compound acting as an antagonist if the activity measured in step (a) is lower in the presence of the compound than in the absence of the compound.

This method can be carried out as described above with regard to screening of agonist compounds.

The pharmaceutical composition of the present invention may further comprise a pharmaceutically acceptable carrier and/or diluent. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions

can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical, intradermal, intranasal or intrabronchial administration. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. A typical dose can be, for example, in the range of 0.001 to 1000 µg (or of nucleic acid for expression or for inhibition of expression in this range); however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{12} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the pharmaceutical composition of the invention may comprise further agents such as interleukins or interferons depending on the intended use of the pharmaceutical composition.

In a further aspect, the present invention relates to the use of any of the compounds as described above for the preparation of a pharmaceutical composition for preventing or treating a pathological condition in which endothelial cells are involved or affected.

The term "endothelial cells" denotes cells which line and form the inner wall of all blood and lymphatic vessels. Endothelial progenitor cells on the other hand can be inside tissues, e.g. in the bone marrow or in circulation, and can be defined as endothelial cells by their ability to incorporate in blood vessels.

The term "pathological condition in which endothelial cells are involved or affected" refers in this context to any acute or chronic pathological condition, wherein endothelial cells are altered compared to the healthy state, said alteration may include cytological features, such as subcellular structure, cell morphology, the expression level(s) of one or more genes, physiological activity, molecular features such as the presence or absence of cellular markers at the cellular surface and/or proliferative activity.

Preferably this use refers to pathological conditions which are accompanied by an altered gene expression in endothelial cells compared to endothelial cells of a healthy subject. More preferred are pathological conditions wherein such an altered gene expression concerns genes the expression of which is regulated by cAMP and/or retinoic acid (RA), in particular on the transcriptional level which can, for example, be measured by determining the amount of specific RNA in the cells. Examples for cAMP and RA-dependent genes are given in Hatzopoulos (Development 125 (1998), 1457-1468) or in the Examples.

In a preferred embodiment, the above mentioned use refers to pathological conditions that can be treated by inhibiting or promoting angiogenesis.

As outlined above, the present invention is based on the observation that in B-Raf KO cells as well as in B-Raf deficient mouse embryos the expression of a lot of genes is altered compared to wild-type cells or embryos. One such alteration concerns genes which are induced in wild-type endothelial cells during angiogenesis such as Flk-1 which encodes the receptor for the vascular endothelial growth factor (VEGF) and the serine/theorine kinase receptor Alk-1. Expression of these genes is

significantly down-regulated in Raf KO cells and embryos. Consequently, modulators of B-Raf activity may be useful for inhibiting or promoting angiogenesis. Specifically, those of the above described compounds that are suited for activating B-Raf, i.e. polynucleotides, vectors, host cells, polypeptides or agonists, in the following also referred to as "activating compounds", may be useful for promoting angiogenesis. On the other hand, those of the above compounds which are antagonists, i.e. being capable of inhibiting B-Raf activity, may be useful for inhibiting angiogenesis.

A further group of genes having an altered expression in B-Raf KO cells and embryos comprises genes involved in apoptosis such as bcl-2. Apoptosis-related genes may also be used for modulating, preferably for inhibiting angiogenesis. For instance, bcl-2 is an anti-apoptosis acting gene. Bcl-2 activity prevents apoptosis in mammalian cells. Bcl-2 interacts with Raf proteins and guides them to the mitochondrial membrane. Inhibition of Raf can then induce endothelial-specific apoptosis that can be used to stop angiogenesis by selectively killing activated endothelial cells. All the diseases that angiogenesis is involved in could be treated by inducing endothelial apoptosis in the angiogenic areas.

Accordingly, in a further preferred embodiment, inhibiting or promoting of angiogenesis may be useful for preventing or treating tumors, diabetic retinopathy, chronic inflammatory diseases, such as psoriasis or arthritis, atherosclerosis or for promoting wound healing or for enhancing circulation, e.g. in conditions where circulation is impaired such as for example after heart infarct, strokes or in the extremities of older people.

Preferably, the inhibitory effect on angiogenesis triggered by antagonist compounds may be used to prevent or treat tumors, treat chronic inflammatory reactions such as psoriasis and arthritis, prevent blindness in diabetic retinopathy and to stop formation and growth of atherosclerotic plaques.

Also preferred is to use the activating compounds that are capable of promoting angiogenesis for restoring blood supply to myocardium after a heart infarct, restoring blood supply to brain after stroke, enhance circulation especially in the extremities of older people and enhance wound healing following injury.

In a further preferred embodiment, the invention refers to the above mentioned uses which are for preventing or treating inflammation or atherosclerosis.

Another group of genes the transcription and expression of which is altered in B-Raf KO cells and embryos are genes involved in endothelial-endothelial cell interactions (e.g. VE-cadherin) or endothelial-immune system cell interactions (e.g. V-CAM-1, E-selectin). These proteins are involved in the interaction of endothelial cells with leukocytes and are instrumental in recruiting these cells during inflammatory conditions. The same genes are also activated in injured endothelium during atherosclerosis. In a similar fashion, it can be expected that modulating B-Raf could lead to therapeutic intervention during inflammation and atherosclerosis.

An additional preferred embodiment relates to the uses mentioned above wherein said pathological condition can be treated by modulating the permeability of the blood-brain barrier.

Genes that control endothelial cell-cell adhesion such as VE-cadherin, E-cadherin and possibly the E-cadherin repressor factor Snail, are important for maintaining the blood-brain barrier. Therefore, modulation of Raf activity could either weaken endothelial cell adhesion and potentially circumvent the barrier for pharmacological administration of brain drugs, or strengthen the barrier to prevent toxic compound penetration to the brain tissue.

Another preferred embodiment refers to uses wherein the pathological condition can be treated by blocking or enhancing endothelial cell migration during angiogenesis or tissue remodeling.

The term "tissue remodeling" used herein refers to growth, differentiation, migration and reorganization of connective and epidermal tissue (fibroblasts, keratinocytes), blood cells (macrophages, lymphocytes, mast cells) and endothelial cells in order to heal an injured area.

The present preferred embodiment is based on the finding that another group of genes having an altered gene expression in B-Raf KO cells and embryos comprise genes involved in endothelial cell-extracellular matrix interactions, such as the gene encoding laminin gamma1, Tie-1 and TGF beta receptor II. Modulators of B-Raf could therefore block or enhance endothelial cell migration or wound healing.

Moreover, based on the altered gene expression of BMP1 and TIMP2 in B-Raf KO embryos and null cells, modulators of B-Raf can be used for inhibiting tumor development, in particular by stopping tumor cell invasion during metastasis.

Furthermore, another preferred embodiment relates to the above uses which are for treating pathophysiological conditions or injury of the vascular wall.
The term "vascular wall" refers to the inner cellular surface of blood and lymphatic vessels.
The term "pathophysiological conditions or injury" of the vascular wall refers to formation of atheriosclerotic plaques and may for example include diseases such as atherosclerosis, high cholesterol levels, abnormal lipid metabolism or smoking addiction and to vascular injury due to striping away of endothelial cells following catheterization, for instance during balloon angioplasty.
A further group of genes having an altered expression in B-Raf KO cells and embryos comprises genes involved in atherosclerosis and oxidative stress such as ApoE, plasma glutathione peroxidase, glutathione S transferases, PDGF receptors alpha and beta, and eNOS. Modulators of B-Raf could have a beneficial effect in protecting the vascular wall during pathophysiological conditions and injury. Besides the endothelial cells, such molecules could be used for treating diseases such as Alzheimer's where abnormal ApoE expression is one cause for the disease.

In a further aspect the invention relates to uses which are for preventing coagulation or fibrin deposition in the vessels.
This embodiment is based on the observation that a further group having an altered expression in B-Raf KO cells and embryos comprise genes involved in thrombosis and coagulation such as von Willebrand Factor (vWF), thrombomodulin etc. Modulators of B-Raf could thus have a beneficial effect in preventing coagulation and fibrin deposition.

Accordingly, in a further preferred embodiment said uses refer to preventing or treating stroke or myocardial infarction.

Additionally, a further preferred embodiment relates to uses which are for preventing or treating restenosis.

Further genes that were revealed to have an altered gene expression in B-Raf KO cells and embryos refer to genes involved in new functions in endothelial cells that are not sufficiently understood at the moment. For example, the expression of a number of wnt genes, their receptors and downstream wnt signaling molecules is deregulated. Although, no wnt gene functions are known in endothelial cells, a recent publication (Dennis, J. Cell Sci. 112 (1994), 3815-3820; Mao, Arterioscler. Thromb. Vasc. Biol. 20 (2000), 43-51) showed that some of the wnt receptors are induced in endothelial and smooth muscle cells during injury of endothelial cells following balloon angioplasty of occluded blood vessels. Since the major problem of this treatment is the occurring restenosis of the affected blood vessels, B-Raf modulators could prevent restenosis.

In a further embodiment, the present invention relates to the above uses, which are for preventing or treating cardiomyopathy.

A further group of genes having an altered expression in B-Raf KO cells and embryos comprises genes encoding proteins involved in heart development such as GATA-4 and 6. These two proteins are essential for heart development and are involved in myocardium differentiation. GATA-4 and 6 inhibitors or activators might be used to treat cardiomyopathies since they are known to regulate transcription of genes such as cardiac myosin heavy chain, troponin I etc. (Charron and Nemer, Cell and Dev. Biol. 10 (1999), 85-91).

In a further aspect, the present invention refers to a method for screening compounds to identify those which act as agonists or antagonists of the polypeptide as defined above, comprising the steps of

(a) measuring the activity of said polypeptide in the presence and in the absence of the compound to be tested; and

(b) determining that the activity measured in step (a) is

(i) higher in the presence of the compound than in the absence of the compound, thereby identifying a compound acting as an agonist;

or

(ii) lower in the presence of the compound than in the absence of the compound, thereby identifying a compound acting as an antagonist.

The conditions under which such a screening method can be conducted have already been described above.

Furthermore, the invention relates to a method for screening compounds to identify those which act as agonists or antagonists of a polypeptide as defined in above, comprising the steps of

(a) incubating cells which are transfected with a polynucleotide as defined above and express said polynucleotide in the presence and in the absence of a compound to be tested;

(b) measuring a specific gene expression in the cells of (a); and

(c) determining that the specific gene expression measured in step (b) is

(i) higher in the presence of the compound than in the absence of the compound, thereby identifying a compound acting as an agonist; or

(ii) lower in the presence of the compound than in the absence of the compound, thereby identifying a compound acting as an antagonist.

The term "specific gene expression" refers to the expression of the genes identified in Table 1. For screens of small numbers of pre-selected compounds, RNA will be isolated and analyzed by RT-PCR as described in the methods below. For high throughput screens, cells may be employed where a gene of interest has been replaced by homologous recombination with an easily quantifiable marker such as bacterial beta-galactosidase. For example, in connection with the invention, cells have been produced where one Flk-1 allele is replaced by beta-galactosidase (knock-in strategy). When Flk-1 is induced or repressed by Raf modulator, the levels of bacterial beta-galactosidase will change correspondingly. Alternatively and for gene targets where no knock-in is available, constructs can be engineered with the beta-galactosidase gene under the control of relevant gene promoter elements and embryonic endothelial cells transfected therewith.

A further aspect of the present invention relates to a method for the production of a pharmaceutical composition comprising the steps of one of the above-described

methods for screening compounds and furthermore the step of formulating the identified compound in a pharmaceutically acceptable form.

The step of formulating a compound in a pharmaceutical acceptable form so as to obtain a pharmaceutical composition of the present invention have already been described in detail above.

Additionally, the present invention relates to a diagnostic composition comprising a polynucleotide as defined above, a host cell genetically engineered with said polynucleotide or a vector comprising said polynucleotide, a polypeptide encoded by said polynucleotide or an antibody specifically recognizing said polypeptide.

The diagnostic composition optionally comprises suitable means for detection. The (poly)peptides and antibodies described above are, for example, suited for use in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. Examples of immunoassays which can utilize said (poly)peptide are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA), the sandwich (immunometric assay) and the Western blot assay. The (poly)peptides and antibodies can be bound to many different carriers and used to isolate cells specifically bound to said polypeptides. Examples of well-known carriers include glass, polystyrene, polyvinyl chloride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for the purposes of the invention.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds; see also the embodiments discussed hereinabove.

Said diagnostic compositions may also be used for methods for detecting expression of a gene encoding a Raf protein by detecting the presence of mRNA coding for a membrane bound fusion protein which comprises obtaining mRNA from a cell and contacting the mRNA so obtained with a probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a nucleic acid molecule

as described above under suitable hybridizing conditions (see also supra), detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of the fusion protein by the cell.

Furthermore, the host cells described above may act as biosensors of early disease signs. When transplanted into a subject, such cells may monitor pathologically high or low Raf activity. Methods to construct corresponding transfected cells which allow for in vivo monitoring are well known to the person skilled in the art.

The components of the diagnostic composition of the present invention may be packaged in containers such as vials, optionally in buffers and/or solutions. If appropriate, one or more of said components may be packaged in one and the same container. Additionally or alternatively, one or more of said components may be adsorbed to a solid support such as, e.g., a nitrocellulose filter or nylon membrane, or to the well of a microtitre-plate.

Furthermore, the present invention refers in another aspect to a method for diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising the steps of

(a) determining the expression level of a polypeptide as defined above in a sample comprising endothelial cells or a lysate thereof; and

(b) diagnosing a pathological condition or a susceptibility to a pathological condition if the expression level determined in step (a) is altered compared to a standard expression level.

Samples for carrying out this method may be taken according to routine clinical procedures known in the art. For instance, during by-pass operations leg veins are removed and are used for heart by-pass operations. Portions of these vains can be used for endothelial isolation. Peripheral arteries can also be removed and used, e.g., for endothelial RNA preparation. In addition, tumor specimens contain activated endothelium. Most frequently though, umbilical veins are used for isolating and growing endothelial cells.

These and other embodiments are disclosed and obvious to a skilled person and embraced by the description and the examples of the present invention. Additional literature regarding one of the above-mentioned methods, means and applications,

which can be used within the meaning of the present invention, can be obtained from the state of the art, for instance from public libraries for instance by the use of electronic means. This purpose can be served inter alia by public databases, such as the "medline", which are accessible via internet, for instance under the address http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Other databases and addresses are known to a skilled person and can be obtained from the internet, for instance under the address http://www.lycos.com. An overview of sources and information regarding patents and patent applications in biotechnology is contained in Berks, TIBTECH 12 (1994), 352-364.

All of the above cited disclosures of patents, publications and database entries are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication or entry were specifically and individually indicated to be incorporated by reference.

Figure 1 shows examples of genes that are deregulated in B-Raf null cells. The amount of specific mRNA has been determined in wild-type endothelial cells ("wild-type") and B-Raf null cells ("KO") using RT-PCR. For each gene, gene expression was measured with (+) and without (-) prior addition of cAMP and retinoic acid (RA) according to the protocol outlined in Hatzopoulos (Development 125 (1998), 1457-1468). As a conclusion from the results, the expression of the genes shown, in particular the cAMP and RA-mediated gene expression, is impaired in B-Raf deficient endothelial cells.

Figure 2 shows that cAMP-regulated genes are downregulated in B-Raf KO embryos whereas genes that are not regulated by cAMP and whose expression is not affected in B-Raf KO cells is also not altered in B-Raf KO embryos. The match between in vitro and in vivo data suggests that B-Raf is a main regulator of these genes in endothelial cells.

Figure 3 shows that cAMP-induced activation of the MAPK pathway in endothelial cells requires B-Raf. For detailed explanations see Example 3.

Figure 4 shows electron micrographs from mouse embryos at day 12.5. These cross-sections show that B-Raf is required for the proper development of peri-endothelial structures. Further explanations are given in Example 4. The dots in the arrows in the two upper panels mark the areas of the smooth muscle cells. The arrows point to the endothelial cells. The arrows in the bottom panels point to collagen fibers in the peri-vascular areas.

Table 1 shows target genes of the B-Raf signaling pathway

The following Examples illustrate the invention.

Experimental setup

Isolation of B-Raf null angioblasts

Mouse embryonic endothelial progenitor cells or angioblasts were isolated as previously described (Hatzopoulos, Development 125 (1988), 1457-1468). To this end, a timed pregnancies from B-Raf heterozygote mice was set up and embryos were isolated at day 7.5 for tissue culture. The mice are of a Black C57 background that are bred in our own animal facility. The original breeding pairs were kindly provided by Dr. Andreas Zimmer from NIH, Bethesda, Maryland. Each embryo was dissociated with trypsin and cultured separately on a feeder layer of mouse embryonic fibroblasts using ESCM 20% as medium (Hatzopoulos, Development 125 (1988), 1457-1468). After several months, six independent angioblast cell lines were established, each derived from a single embryo. Genomic DNA was isolated from all lines by lysing cells O/N at 55°C in 400µl 50mM KCl, 1.5mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20 and 100µg/ml proteinase K. The lysates were heat-inactivated at 95°C for 20 minutes, and spun at 13,000 rpm in a table microfuge. The clear supernatant was collected and used for genotyping. Genotyping was performed by PCR using primer pairs that can distinguish between the wild type and the knocked out allele. Following oligonucleotides were used for genotyping:

1. mBraf3-1

5' - GCC TAT GAA GAG TAC ACC AGC AAG CTA GAT GCC C – 3' (SEQ ID NO: 7);

2. mrafB-des
   5' – TAG GTT TCT GTG GTG ACT TGG GGT TGT TCC GTG A – 3' (SEQ ID NO: 8); and
3. Neo-L
   5' – AGT GCC AGC GGG GCT GCT AAA – 3' (SEQ ID NO: 9).

Primers 1 and 2 amplify the wt allele and primers 1 and 3 amplify the KO allele.The PCR contained dNTPs each at 0.22mM, 1X buffer B (PROMEGA), gene-specific primers 0.5µM each, 2µl of genomic DNA and 0.6 units Taq polymerase. The PCR program was 1 minute 95°C, 1 minute 58°C, 2 minutes 72°C for 30 cycles. The reactions were given a final incubation of 10 minutes at 72°C and cooled to 4°C. The PCR products were separated on 1.5% agarose gels, the gels were stained with EtBr and photographed.
The results showed that one line lacked both wild type (wt) B-Raf alleles and it was therefore a null B-Raf endothelial cell line. The B-Raf null cells were frozen in liquid nitrogen.

Example 1: Gene expression profile analysis of wt and B-Raf null endothelial cells

In order to investigate the function of B-Raf in endothelial cells, gene expression profiles between the two different cell types were analyzed using commercially available cDNA arrays (CLONTECH) following the manufacturer's specifications. A number of the observed differences were selected for further confirmation by RNA analysis using RT-PCR. To this end, RNA was isolated from wt and B-Raf null cells with the QIAGEN RNeasy kit. 1µg of total RNA was annealed to 100ng of oligo$(dT)_{15}$ at 65°C for 5 minutes. Then, dNTPs (final concentration 1mM each), reaction buffer (final concentration 60mM KCl, 15mM Tris-Cl pH 8.4, 3mM $MgCl_2$, 0.3% Tween 20, 5mM DTT, 5mM β-MSH, 10 units RNasin) and 100 units Mo-MLV Reverse Transcriptase (LifeTechnologies) was added. First strand synthesis was then

performed at 37°C for 55 minutes. The reaction was stopped by heat inactivation at 95°C for 5 minutes.

The prepared cDNA was used as template in PCR reactions in order to analyze and compare gene expression profiles in wt and B-Raf null cells. The PCR conditions were as follows: dNTPs each at 0.22mM, 5% DMSO, 1X buffer B (PROMEGA) 50.6mM KCl, 1.53mM $MgCl_2$, gene-specific primers 0.5µM each, 10ng cDNA and 0.6 units Taq polymerase). The PCR program was 1 minute 95°C, 1 minute 65°C, 1 minute 72°C for 30 cycles. After cycling, the reactions were incubated for 5 minutes at 72°C and cooled to 4°C. Amplification products were separated on 1.5% agarose gels, the gels were stained with EtBr and photographed.

The results showed that a number of genes are deregulated, i.e. having an altered gene expression, in endothelial cells following gene inactivation of B-Raf (Figure 1). It was also discovered that genes that are normally induced by cAMP and retinoic acid (RA) in wt angioblasts (Hatzopoulos, Development 125 (1998), 1457-1468) fail to do so in the B-Raf null cells (Figure 1). These results are consistent with the role of the B-Raf protein kinase as a downstream molecule in the cAMP-signaling pathway. B-Raf is a direct target of Protein Kinase A (PKA) which in turn is activated by cAMP. Consequently, the cAMP-signaling pathway is impaired in the B-Raf null cells. Table 1 provides a list of some of the genes that were shown to have an up or down-regulated gene expression in B-Raf null cells.

Table 1

GATA-4
GATA-6
VWF
Flk-1
Alk-1
ENOS
Thrombomodulin
V-CAM1
VE-cadherin
E-Selectin
Tie-1
PDGF receptor alpha
PDGF receptor beta
Laminin gamma 1
Wnt (wnt11)
Wnt signaling pathway genes (disheveled, groucho, frizzled receptors)
TGF beta receptor II
Bcl2
Snail
E-cadherin
Plasma glutathione peroxidase (GPX)
Glutathione S transferase (GST)
ApoE
BMP1
TIMP2

Example 2: Endothelial gene expression repertoire is altered in B-Raf deficient mice

In subsequent experiments, the results obtained in B-Raf null cells in tissue culture were confirmed by comparing gene expression profiles between wt and B-Raf KO embryos in vivo. To this end, timed pregnancies from B-Raf heterozygote mice and isolated embryos were set up at day 10.5. A part from each embryo was used for genomic DNA preparation and genotyping as described above. The remaining tissue from each embryo was processed for RNA isolation with the RNeasy QIAGEN kit. RNA from wt and B-Raf KO embryos was then used for RT-PCR analysis as described above.

The thus obtained results showed that genes, whose expression changed in the B-Raf null cells, were similarly affected during embryonic vascular development. In brief, genes that were down regulated in null cells in tissue culture, and/or failed to be induced by cAMP and retinoic acid (RA), were also markedly reduced in the B-Raf deficient embryos (Figure 2).

Example 3: B-Raf kinase cascade assay with wt and B-Raf null embryonic endothelial progenitor cells (eEPC)

Wild type or B-Raf KO eEPCs were grown in 10 cm tissue culture plates to a confluence of 80 % and starved in 0.1 % BSA-DMEM for 18 h. The cells were subsequently stimulated for 5 or 10 minutes with 0.1 % BSA-DMEM containing 0.5mM cAMP. In control reactions the 0.5mM cAMP was omitted. Following stimulation, the cells were lysed in 800 µl Lysis buffer per plate.

(Lysis buffer: 20 mM Tris pH 7.5
5 mM $MgCl_2$
10 mM EGTA
150 mM NaCl
50 mM NaF
40 mM $Na_2P_2O_7$
1 mM Na_3VO_4
1 % Triton
1 mM PMSF, Aprotinin, Leupeptin)

The cells were incubated for 10 min on ice and the lysates were centrifuged at 12,000 rpm, 15 min at 4°C and protein concentration was determined in the supernatant using the BIORAD protein assay kit. 500µg of protein lysate were pre-cleared with addition of 1/10 Volume of Protein-G-Sepharose and incubated for 10 min at 4°C. The samples were centrifuged at 12,000 rpm, 10 min, 4°C. The B-Raf protein was immunoprecipitated (IP) as follows: 2 µg/ml of Anti-B-Raf antibody (from St. Cruz Biotechnology, St. Cruz, CA, USA) were added and the lysates were incubated for 2 hours at 4°C. Subsequently, 50µl of 50% beads-slurry of Protein-G-Sepharose (in Lysis buffer or PBS) were added and incubated for another hour. The beads were washed 3x in lysis buffer without protease inhibitors (PMSF, Aprotinin, Leupeptin) at 4°C and resuspended in immunoprecititation buffer (1% NP-40, 0.5% sodium dioxycholate, 0.1% SDS in PBS).

B-Raf activity was measured using the B-Raf kinase kit from Upstate Biotechnologies: ADB and Mg/ATP-Mix were thawed rapidly, mixed thoroughly and placed on ice. A Master-Mix was prepared on ice as follows:

10 µl Mg/ATP-Mix
1.6 µl MEK1, inactive
4 µl MAP Kinase 2/Erk2
2 µl [γ-^{32}P]-ATP-solution
22.4 µl ADB (total volume per reaction 40 µl)

40 µl of Master-Mix was dispensed to each tube containing the B-Raf immunoprecipitate (on ice), mixed well, and the tubes were transferred to a shaker at 30°C. As positive control 1 µl of activated B-Raf protein (provided with the kit) was used. The reactions were incubated for 30 min and terminated by transferring on ice and subsequent addition of Laemmli loading buffer. The samples were boiled for 5 min at 95°C and analysed on a standard polyacrylamide gel. The gel was blotted on a PVDF-Membrane and B-Raf activity was detected by autoradiography and visualization of phosphorylated MEK1 and MAPK2 (ERK2) (Figure 3).

As it is shown in Figure 3, cAMP can induce activation of the MAP kinase pathway in eEPCs as it is apparent from the phosphorylation of MEK1 and MAPK2 (also called ERK2). This activation fails to occur in eEPCs lacking B-Raf. These results suggest that the effects of a number of divers extracellular signals that modulate intracellular

cAMP levels in endothelial cells, and thus lead to MAPK pathway activation, can in turn be modulated by agents that affect B-Raf activity.

Example 4: Electron microscopy analysis of wild type and B-Raf KO embryos

A pair of B-Raf heterozygote mice was mated and embryos were isolated at day 12.5. The extra-embryonic yolk sac membrane was placed in DNA lysis buffer and genotyped for identification of wild type, heterozygote and homozygote embryos (the genotyping protocol has been described above in connection with the isolation of B-Raf null angioblasts). The corresponding embryos were individually fixed immediately upon isolation in 2-4% ice-cold paraformaldehyde in PBS O/N. Following genotyping and identification, wild type and homozygote embryos were paraffin embedded and sectioned. Sections were then analyzed using electron microscopy (Figure 4).
Electron microscopy of wild type and KO embryos revealed that B-Raf is important for the proper establishment of the endothelial / peri-endothelial environment. Blood vessels in B-Raf KO embryos, such as the dorsal aorta, display a highly disorganized smooth muscle around the inner endothelial cell layer (Figure 4). It was also evident that the KO tissues had dramatically reduced collagen fibers around the blood vessels. This phenotype is strikingly similar to the phenotype of the angiopoietin 1 (Ang1) KO mice (Suri, Cell 87 (1996), 1171-1180). Ang1 is a ligand of the endothelial-specific tyrosine kinase receptor tie-2. Tie-2 is critical for endothelial cell interactions with peri-vascular cells such as smooth muscle cells and it is involved in angiogenesis, wound healing and vascular remodeling (Dumont, Gen. Develop. 8 (1994), 1897-1909; Suri, Science 282 (1998), 468-471; Papapetropoulos, J. Biol. Chem. 275 (2000), 9102-9105). The findings presented herein indicate that B-Raf is downstream from tie-2 and thus further substantiate the role of B-Raf in angiogenesis, wound healing and endothelial cell migration.

CLAIMS

1. A pharmaceutical composition comprising a compound selected from the group consisting of
   (i) polynucleotides encoding a Raf protein selected from the group consisting of
      (a) polynucleotides encoding a polypeptide comprising the amino acid sequence of any one of SEQ ID NOs: 2, 4 and 6;
      (b) polynucleotides comprising the coding region of the nucleotide sequence of any one of SEQ ID NOs: 1, 3 and 5;
      (c) polynucleotides encoding a fragment of a polypeptide encoded by a polynucleotide of (a) or (b); and
      (d) polynucleotides the complementary strand of which hybridizes with a polynucleotide of any one of (a) to (c) and encode a polypeptide having Raf activity;
   (ii) vectors comprising and capable of expressing said polynucleotide;
   (iii) host cells genetically engineered with said polynucleotide or said vector;
   (iv) polypeptides encoded by said polynucleotide; and
   (v) agonists of a polypeptide encoded by said polynucleotide;
   and optionally a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1, wherein the Raf protein is B-Raf.

3. A pharmaceutical composition comprising a compound which is an antagonist of the polypeptide encoded by a polynucleotide as defined in claim 1 or 2 and optionally a pharmaceutically acceptable carrier.

4. The pharmaceutical composition of claim 1 or 2, wherein the compound which is an agonist can be identified by a method for screening such compounds comprising the steps of
   (a) measuring the activity of said polypeptide in the presence and in the absence of the compound to be tested; and

(b) identifying a compound acting as an agonist if the activity measured in step (a) is higher in the presence of the compound than in the absence of the compound.

5. The pharmaceutical composition of claim 3, wherein the compound which is an antagonist can be identified by a method for screening such compounds comprising the steps of

(a) measuring the activity of said polypeptide in the presence and in the absence of the compound to be tested; and

(b) identifying a compound acting as an antagonist if the activity measured in step (a) is lower in the presence of the compound than in the absence of the compound.

6. Use of a compound as defined in any one of claims 1 to 5 for the preparation of a pharmaceutical composition for preventing or treating a pathological condition in which endothelial cells are involved or affected.

7. The use of claim 6, wherein the pathological condition can be treated by inhibiting or promoting angiogenesis.

8. The use of claim 6 or 7, which is for preventing or treating tumors, diabetic retinopathy or chronic inflammatory disease or for promoting wound healing or for enhancing circulation.

9. The use of claim 6, which is for preventing or treating inflammation or atherosclerosis.

10. The use of claim 6, wherein the pathological condition can be treated by modulating the permeability of the blood-brain barrier.

11. The use of claim 6, wherein the pathological condition can be treated by blocking or enhancing endothelial cell migration during angiogenesis or tissue remodeling.

12. The use of claim 6, which is for inhibiting tumor development.

13. The use of claim 12, wherein said tumor development is metastasis.

14. The use of claim 6, which is for treating pathophysiological conditions or injury of the vascular wall.

15. The use of claim 6, which is for treating Alzheimer's disease.

16. The use of claim 6, which is for preventing coagulation or fibrin deposition in the vessels.

17. The use of claim 6 or 16, which is for preventing or treating stroke or myocardial infarction.

18. The use of claim 6, which is for preventing or treating restenosis.

19. The use of claim 6, which is for preventing or treating cardiomyopathy.

20. A method for screening compounds to identify those which act as agonists or antagonists of the polypeptide as defined in claim 1 or 2, comprising the steps of

(a) measuring the activity of said polypeptide in the presence and in the absence of the compound to be tested; and

(b) determining that the activity measured in step (a) is

(i) higher in the presence of the compound than in the absence of the compound, thereby identifying a compound acting as an agonist; or

(ii) lower in the presence of the compound than in the absence of the compound, thereby identifying a compound acting as an antagonist.

21. A method for screening compounds to identify those which act as agonists or antagonists of a polypeptide as defined in claim 1 or 2, comprising the steps of

(a) incubating cells which are transfected with a polynucleotide as defined in claim 1 or 2 and express said polynucleotide in the presence and in the absence of a compound to be tested;

(b) measuring a specific gene expression in the cells of (a); and

(c) determining that the specific gene expression measured in step (b) is

(i) higher in the presence of the compound than in the absence of the compound, thereby identifying a compound acting as an agonist; or

(ii) lower in the presence of the compound than in the absence of the compound, thereby identifying a compound acting as an antagonist.

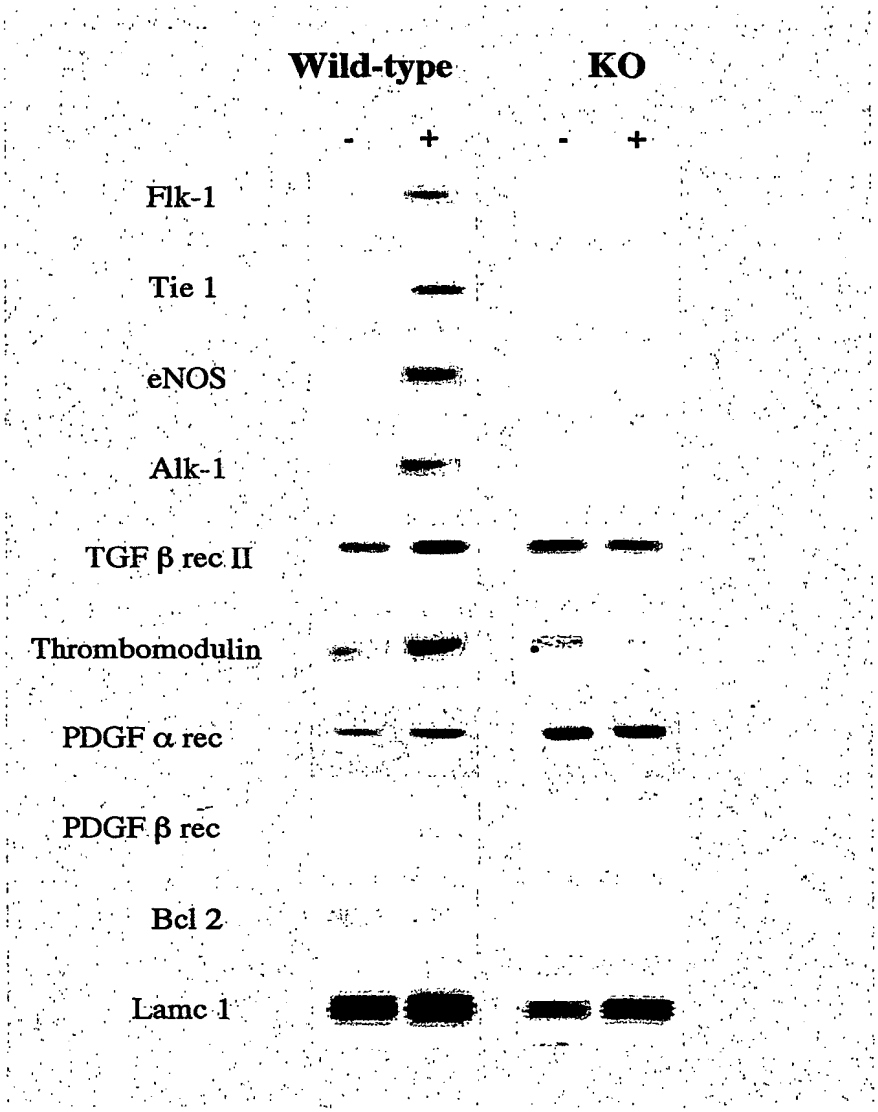
22. A method for the production of a pharmaceutical composition comprising the steps of the method of claim 20 or 21 and furthermore the step of formulating the identified compound in a pharmaceutically acceptable form.

23. A diagnostic composition comprising the polynucleotide as defined in claim 1 or 2, a host cell genetically engineered with said polynucleotide or a vector comprising said polynucleotide, a polypeptide encoded by said polynucleotide or an antibody specifically recognizing said polypeptide.

24. A method for diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising the steps of

(a) determining the expression level of a polypeptide as defined in claim 1 or 2 in a sample comprising endothelial cells or a lysate thereof; and

(b) diagnosing a pathological condition or a susceptibility to a pathological condition if the expression level determined in step (a) is altered compared to a standard expression level.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+
-
+
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Thrombomodulin
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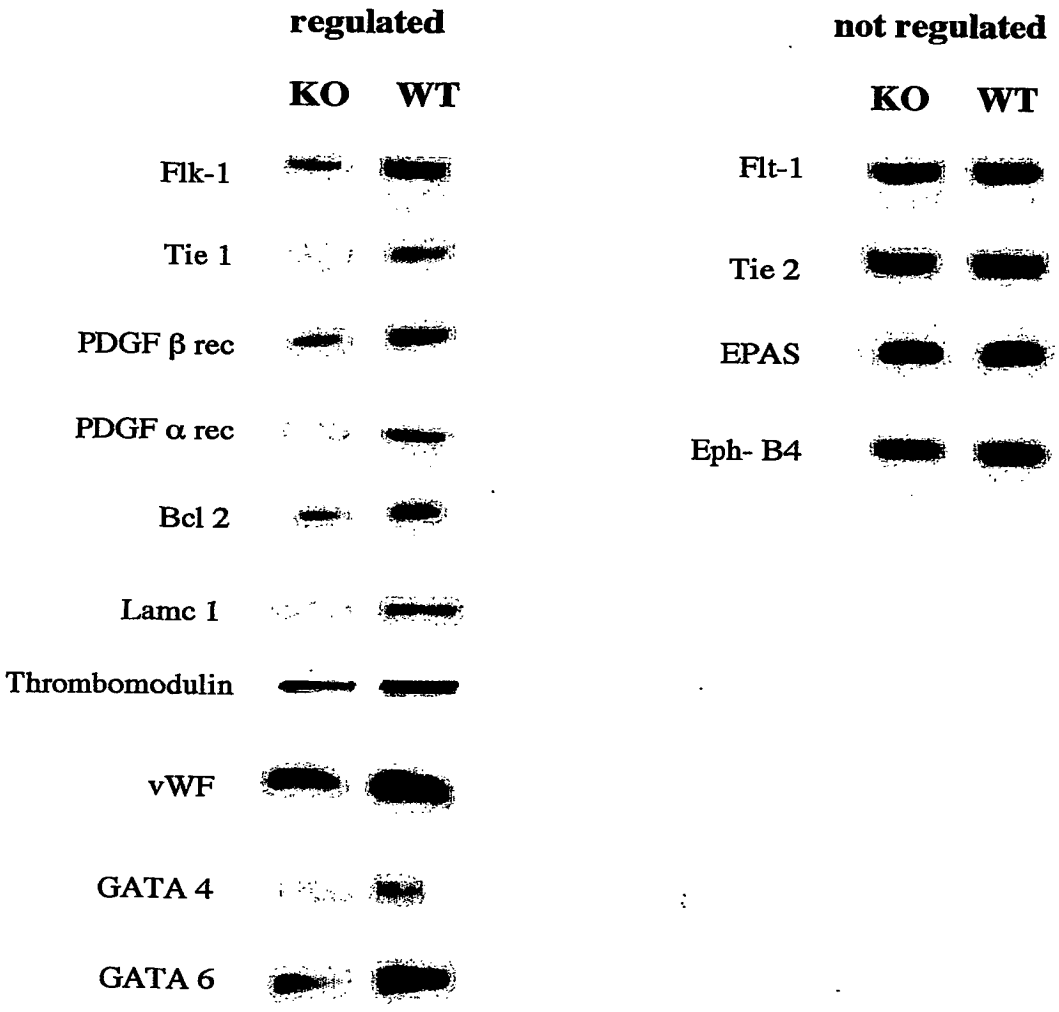
Bcl 2
Lamc 1

Fig. 1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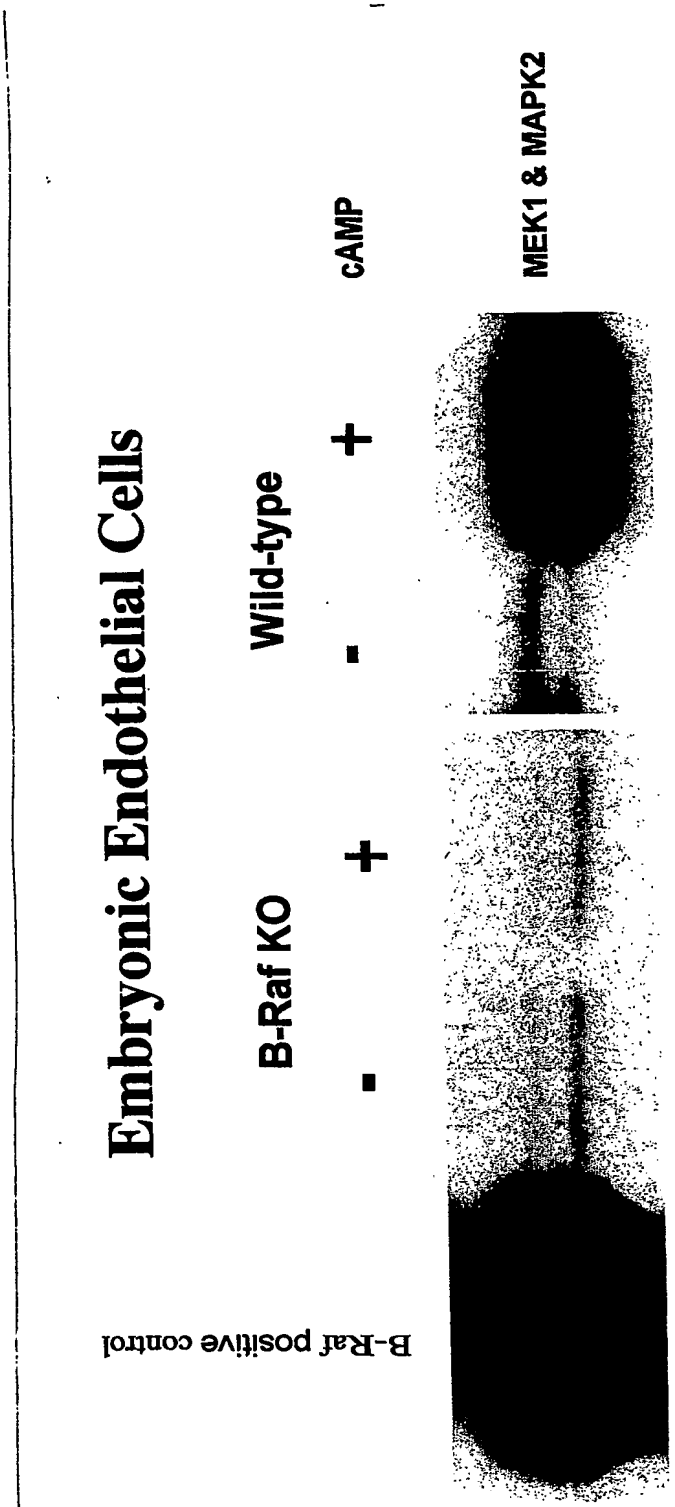
Eph- B4

Fig. 2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+
-
+
-
MEK1 & MAPK2
B-Raf positive control

Fig. 3

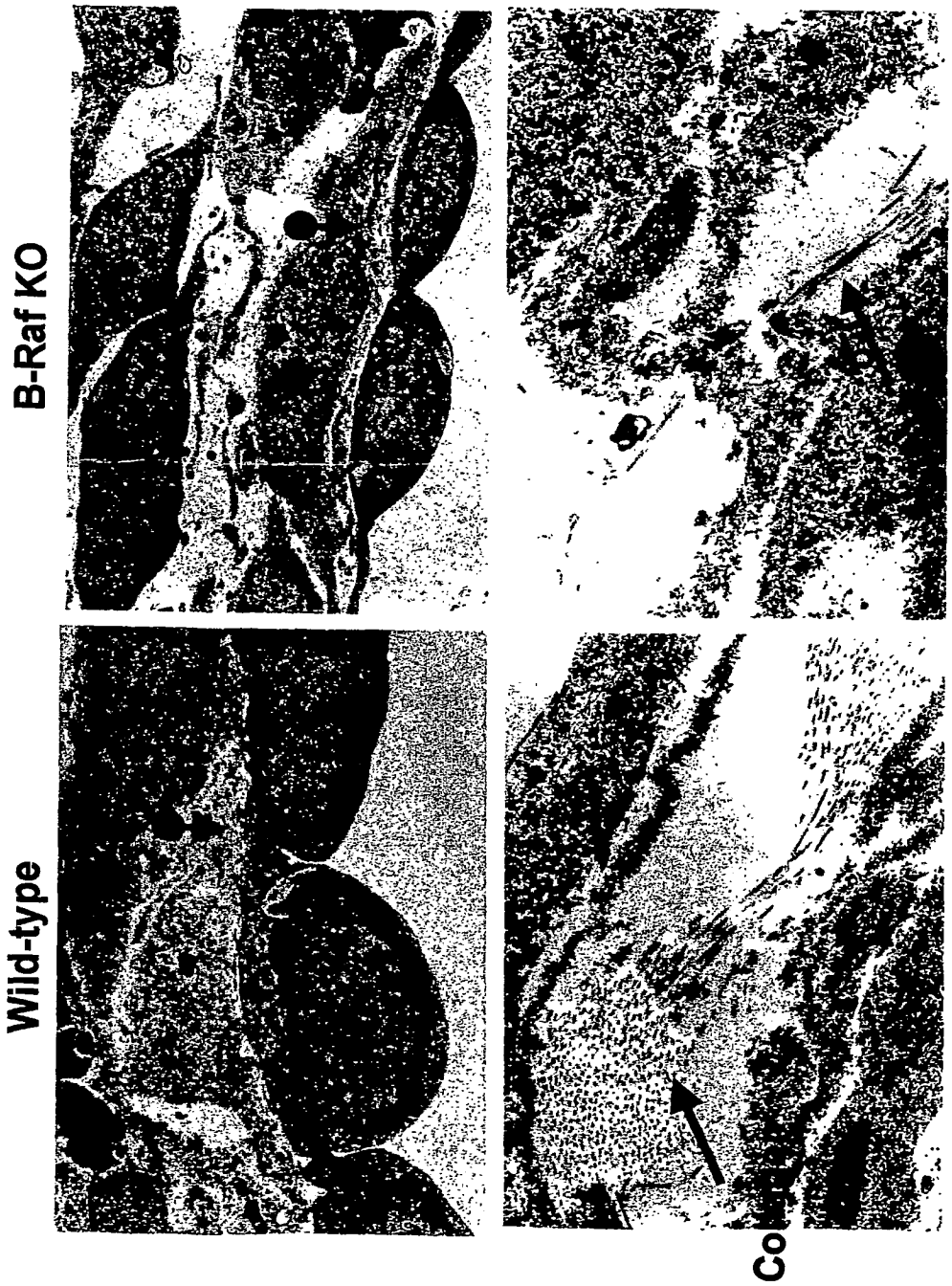

Fig. 4

SEQUENCE LISTING

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Cys Gln Thr Cys Gly Tyr Lys Phe His Gln His Cys Ser Ser Lys Val
125                 130                 135                 140

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            240                 245                 250

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365                 370                 375                 380

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            560                 565                 570

tca ctc ccc aag att gag cgg agt gcc tcg gaa ccc tcc ttg cac cgc   1958
Ser Leu Pro Lys Ile Glu Arg Ser Ala Ser Glu Pro Ser Leu His Arg
        575                 580                 585
```

```
acc cag gcc gat gag ttg cct gcc tgc cta ctc agc gca gcc cgc ctt   2006
Thr Gln Ala Asp Glu Leu Pro Ala Cys Leu Leu Ser Ala Ala Arg Leu
    590                 595                 600

gtg cct tag gccccgccca agccaccagg gagccaatct cagccctcca           2055
Val Pro
605

cgccaaggag ccttgcccac cagccaatca atgttcgtct ctgccctgat gctgcctcag 2115

gatcccccat tccccaccct gggagatgag ggggtcccca tgtgcttttc cagttcttct 2175

ggaattgggg gacccccgcc aaagactgag ccccctgtct cctccatcat ttggtttcct 2235

cttggctttg gggatacttc taaattttgg gagctcctcc atctccaatg gctgggattt 2295

gtggcaggga ttccactcag aacctctctg gaatttgtgc ctgatgtgcc ttccactgga 2355

ttttggggtt cccagcaccc catgtggatt ttgggggtc ccttttgtgt ctcccccgcc 2415

attcaaggac tcctctcttt cttcaccaag aagcacagaa ttc                   2458
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<210> 2
<211> 606
<212> PRT
<213> Homo sapiens

<400> 2

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Met Glu Pro Pro Arg Gly Pro Pro Ala Asn Gly Ala Glu Pro Ser Arg
  1               5                  10                  15
Ala Val Gly Thr Val Lys Val Tyr Leu Pro Asn Lys Gln Arg Thr Val
            20                  25                  30
Val Thr Val Arg Asp Gly Met Ser Val Tyr Asp Ser Leu Asp Lys Ala
        35                  40                  45
Leu Lys Val Arg Gly Leu Asn Gln Asp Cys Cys Val Val Tyr Arg Leu
    50                  55                  60
Ile Lys Gly Arg Lys Thr Val Thr Ala Trp Asp Thr Ala Ile Ala Pro
65                  70                  75                  80
Leu Asp Gly Glu Glu Leu Ile Val Glu Val Leu Glu Asp Val Pro Leu
                85                  90                  95
Thr Met His Asn Phe Val Arg Lys Thr Phe Phe Ser Leu Ala Phe Cys
            100                 105                 110
Asp Phe Cys Leu Lys Phe Leu Phe His Gly Phe Arg Cys Gln Thr Cys
        115                 120                 125
Gly Tyr Lys Phe His Gln His Cys Ser Ser Lys Val Pro Thr Val Cys
    130                 135                 140
Val Asp Met Ser Thr Asn Arg Gln Gln Phe Tyr His Ser Val Gln Asp
145                 150                 155                 160
Leu Ser Gly Gly Ser Arg Gln His Glu Ala Pro Ser Asn Arg Pro Leu
                165                 170                 175
Asn Glu Leu Leu Thr Pro Gln Gly Pro Ser Pro Arg Thr Gln His Cys
            180                 185                 190
Asp Pro Glu His Phe Pro Phe Pro Ala Pro Ala Asn Ala Pro Leu Gln
        195                 200                 205
Arg Ile Arg Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr
    210                 215                 220
Ala Pro Met Asp Ser Asn Leu Ile Gln Leu Thr Gly Gln Ser Phe Ser
225                 230                 235                 240
```

```
Thr Asp Ala Ala Gly Ser Arg Gly Gly Ser Asp Gly Thr Pro Arg Gly
                245                 250                 255
Ser Pro Ser Pro Ala Ser Val Ser Ser Gly Arg Lys Ser Pro His Ser
            260                 265                 270
Lys Ser Pro Ala Glu Gln Arg Glu Arg Lys Ser Leu Ala Asp Asp Lys
        275                 280                 285
Lys Lys Val Lys Asn Leu Gly Tyr Arg Xaa Ser Gly Tyr Tyr Trp Glu
    290                 295                 300
Val Pro Pro Ser Glu Val Gln Leu Leu Lys Arg Ile Gly Thr Gly Ser
305                 310                 315                 320
Phe Gly Thr Val Phe Arg Gly Arg Trp His Gly Asp Val Ala Val Lys
                325                 330                 335
Val Leu Lys Val Ser Gln Pro Thr Ala Glu Gln Ala Gln Ala Phe Lys
            340                 345                 350
Asn Glu Met Gln Val Leu Arg Lys Thr Arg His Val Asn Ile Leu Leu
        355                 360                 365
Phe Met Gly Phe Met Thr Arg Pro Gly Phe Ala Ile Ile Thr Gln Trp
    370                 375                 380
Cys Glu Gly Ser Ser Leu Tyr His His Leu His Val Ala Asp Thr Arg
385                 390                 395                 400
Phe Asp Met Val Gln Leu Ile Asp Val Ala Arg Gln Thr Ala Gln Gly
                405                 410                 415
Met Asp Tyr Leu His Ala Lys Asn Ile Ile His Arg Asp Leu Lys Ser
            420                 425                 430
Asn Asn Ile Phe Leu His Glu Gly Leu Thr Val Lys Ile Gly Asp Phe
        435                 440                 445
Gly Leu Ala Thr Val Lys Thr Arg Trp Ser Gly Ala Gln Pro Leu Glu
    450                 455                 460
Gln Pro Ser Gly Ser Val Leu Trp Met Ala Ala Glu Val Ile Arg Met
465                 470                 475                 480
Gln Asp Pro Asn Pro Tyr Ser Phe Gln Ser Asp Val Tyr Ala Tyr Gly
                485                 490                 495
Val Val Leu Tyr Glu Leu Met Thr Gly Ser Leu Pro Tyr Ser His Ile
            500                 505                 510
Gly Cys Arg Asp Gln Ile Ile Phe Met Val Gly Arg Gly Tyr Leu Ser
        515                 520                 525
Pro Asp Leu Ser Lys Ile Ser Ser Asn Cys Pro Lys Ala Met Arg Arg
    530                 535                 540
Leu Leu Ser Asp Cys Leu Lys Phe Gln Arg Glu Glu Arg Pro Leu Phe
545                 550                 555                 560
Pro Gln Ile Leu Ala Thr Ile Glu Leu Leu Gln Arg Ser Leu Pro Lys
                565                 570                 575
Ile Glu Arg Ser Ala Ser Glu Pro Ser Leu His Arg Thr Gln Ala Asp
            580                 585                 590
Glu Leu Pro Ala Cys Leu Leu Ser Ala Ala Arg Leu Val Pro
        595                 600                 605
```

<210> 3
<211> 2510
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (62)..(2359)

<400> 3
cgcctcccgg ccccctcccc gcccgacagc ggccgctcgg gccccggctc tcggttataa 60

```
g atg gcg gcg ctg agc ggt ggc ggt ggt ggc ggc gcg gag ccg ggc cag 109
  Met Ala Ala Leu Ser Gly Gly Gly Gly Gly Gly Ala Glu Pro Gly Gln
    1               5                  10                  15

gct ctg ttc aac ggg gac atg gag ccc gag gcc ggc gcc ggc cgg ccc   157
Ala Leu Phe Asn Gly Asp Met Glu Pro Glu Ala Gly Ala Gly Arg Pro
            20                  25                  30

gcg gcc tct tcg gct gcg gac cct gcc att ccg gag gag gtg tgg aat   205
Ala Ala Ser Ser Ala Ala Asp Pro Ala Ile Pro Glu Glu Val Trp Asn
        35                  40                  45

atc aaa caa atg att aag ttg aca cag gaa cat ata gag gcc cta ttg   253
Ile Lys Gln Met Ile Lys Leu Thr Gln Glu His Ile Glu Ala Leu Leu
    50                  55                  60

gac aaa ttt ggt ggg gag cat aat cca cca tca ata tat ctg gag gcc   301
Asp Lys Phe Gly Gly Glu His Asn Pro Pro Ser Ile Tyr Leu Glu Ala
65                  70                  75                  80

tat gaa gaa tac acc agc aag cta gat gca ctc caa caa aga gaa caa   349
Tyr Glu Glu Tyr Thr Ser Lys Leu Asp Ala Leu Gln Gln Arg Glu Gln
                85                  90                  95

cag tta ttg gaa tct ctg ggg aac gga act gat ttt tct gtt tct agc   397
Gln Leu Leu Glu Ser Leu Gly Asn Gly Thr Asp Phe Ser Val Ser Ser
            100                 105                 110

tct gca tca atg gat acc gtt aca tct tct tcc tct tct agc ctt tca   445
Ser Ala Ser Met Asp Thr Val Thr Ser Ser Ser Ser Ser Ser Leu Ser
        115                 120                 125

gtg cta cct tca tct ctt tca gtt ttt caa aat ccc aca gat gtg gca   493
Val Leu Pro Ser Ser Leu Ser Val Phe Gln Asn Pro Thr Asp Val Ala
    130                 135                 140

cgg agc aac ccc aag tca cca caa aaa cct atc gtt aga gtc ttc ctg   541
Arg Ser Asn Pro Lys Ser Pro Gln Lys Pro Ile Val Arg Val Phe Leu
145                 150                 155                 160

ccc aac aaa cag agg aca gtg gta cct gca agg tgt gga gtt aca gtc   589
Pro Asn Lys Gln Arg Thr Val Val Pro Ala Arg Cys Gly Val Thr Val
                165                 170                 175

cga gac agt cta aag aaa gca ctg atg atg aga ggt cta atc cca gag   637
Arg Asp Ser Leu Lys Lys Ala Leu Met Met Arg Gly Leu Ile Pro Glu
            180                 185                 190

tgc tgt gct gtt tac aga att cag gat gga gag aag aaa cca att ggt   685
Cys Cys Ala Val Tyr Arg Ile Gln Asp Gly Glu Lys Lys Pro Ile Gly
        195                 200                 205

tgg gac act gat att tcc tgg ctt act gga gaa gaa ttg cat gtg gaa   733
Trp Asp Thr Asp Ile Ser Trp Leu Thr Gly Glu Glu Leu His Val Glu
    210                 215                 220

gtg ttg gag aat gtt cca ctt aca aca cac aac ttt gta cga aaa acg   781
Val Leu Glu Asn Val Pro Leu Thr Thr His Asn Phe Val Arg Lys Thr
225                 230                 235                 240
```

```
ttt ttc acc tta gca ttt tgt gac ttt tgt cga aag ctg ctt ttc cag   829
Phe Phe Thr Leu Ala Phe Cys Asp Phe Cys Arg Lys Leu Leu Phe Gln
                245                 250                 255

ggt ttc cgc tgt caa aca tgt ggt tat aaa ttt cac cag cgt tgt agt   877
Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Gln Arg Cys Ser
            260                 265                 270

aca gaa gtt cca ctg atg tgt gtt aat tat gac caa ctt gat ttg ctg   925
Thr Glu Val Pro Leu Met Cys Val Asn Tyr Asp Gln Leu Asp Leu Leu
        275                 280                 285

ttt gtc tcc aag ttc ttt gaa cac cac cca ata cca cag gaa gag gcg   973
Phe Val Ser Lys Phe Phe Glu His His Pro Ile Pro Gln Glu Glu Ala
    290                 295                 300

tcc tta gca gag act gcc cta aca tct gga tca tcc cct tcc gca ccc   1021
Ser Leu Ala Glu Thr Ala Leu Thr Ser Gly Ser Ser Pro Ser Ala Pro
305                 310                 315                 320

gcc tcg gac tct att ggg ccc caa att ctc acc agt ccg tct cct tca   1069
Ala Ser Asp Ser Ile Gly Pro Gln Ile Leu Thr Ser Pro Ser Pro Ser
                325                 330                 335

aaa tcc att cca att cca cag ccc ttc cga cca gca gat gaa gat cat   1117
Lys Ser Ile Pro Ile Pro Gln Pro Phe Arg Pro Ala Asp Glu Asp His
            340                 345                 350

cga aat caa ttt ggg caa cga gac cga tcc tca tca gct ccc aat gtg   1165
Arg Asn Gln Phe Gly Gln Arg Asp Arg Ser Ser Ser Ala Pro Asn Val
        355                 360                 365

cat ata aac aca ata gaa cct gtc aat att gat gac ttg att aga gac   1213
His Ile Asn Thr Ile Glu Pro Val Asn Ile Asp Asp Leu Ile Arg Asp
    370                 375                 380

caa gga ttt cgt ggt gat gga gga tca acc aca ggt ttg tct gct acc   1261
Gln Gly Phe Arg Gly Asp Gly Gly Ser Thr Thr Gly Leu Ser Ala Thr
385                 390                 395                 400

ccc cct gcc tca tta cct ggc tca cta act aac gtg aaa gcc tta cag   1309
Pro Pro Ala Ser Leu Pro Gly Ser Leu Thr Asn Val Lys Ala Leu Gln
                405                 410                 415

aaa tct cca gga cct cag cga gaa agg aag tca tct tca tcc tca gaa   1357
Lys Ser Pro Gly Pro Gln Arg Glu Arg Lys Ser Ser Ser Ser Ser Glu
            420                 425                 430

gac agg aat cga atg aaa aca ctt ggt aga cgg gac tcg agt gat gat   1405
Asp Arg Asn Arg Met Lys Thr Leu Gly Arg Arg Asp Ser Ser Asp Asp
        435                 440                 445

tgg gag att cct gat ggg cag att aca gtg gga caa aga att gga tct   1453
Trp Glu Ile Pro Asp Gly Gln Ile Thr Val Gly Gln Arg Ile Gly Ser
    450                 455                 460

gga tca ttt gga aca gtc tac aag gga aag tgg cat ggt gat gtg gca   1501
Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp His Gly Asp Val Ala
465                 470                 475                 480
```

```
gtg aaa atg ttg aat gtg aca gca cct aca cct cag cag tta caa gcc   1549
Val Lys Met Leu Asn Val Thr Ala Pro Thr Pro Gln Gln Leu Gln Ala
                485                 490                 495

ttc aaa aat gaa gta gga gta ctc agg aaa aca cga cat gtg aat atc   1597
Phe Lys Asn Glu Val Gly Val Leu Arg Lys Thr Arg His Val Asn Ile
            500                 505                 510

cta ctc ttc atg ggc tat tcc aca aag cca caa ctg gct att gtt acc   1645
Leu Leu Phe Met Gly Tyr Ser Thr Lys Pro Gln Leu Ala Ile Val Thr
        515                 520                 525

cag tgg tgt gag ggc tcc agc ttg tat cac cat ctc cat atc att gag   1693
Gln Trp Cys Glu Gly Ser Ser Leu Tyr His His Leu His Ile Ile Glu
    530                 535                 540

acc aaa ttt gag atg atc aaa ctt ata gat att gca cga cag act gca   1741
Thr Lys Phe Glu Met Ile Lys Leu Ile Asp Ile Ala Arg Gln Thr Ala
545                 550                 555                 560

cag ggc atg gat tac tta cac gcc aag tca atc atc cac aga gac ctc   1789
Gln Gly Met Asp Tyr Leu His Ala Lys Ser Ile Ile His Arg Asp Leu
                565                 570                 575

aag agt aat aat ata ttt ctt cat gaa gac ctc aca gta aaa ata ggt   1837
Lys Ser Asn Asn Ile Phe Leu His Glu Asp Leu Thr Val Lys Ile Gly
            580                 585                 590

gat ttt ggt cta gct aca gtg aaa tct cga tgg agt ggg tcc cat cag   1885
Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp Ser Gly Ser His Gln
        595                 600                 605

ttt gaa cag ttg tct gga tcc att ttg tgg atg gca cca gaa gtc atc   1933
Phe Glu Gln Leu Ser Gly Ser Ile Leu Trp Met Ala Pro Glu Val Ile
    610                 615                 620

aga atg caa gat aaa aat cca tac agc ttt cag tca gat gta tat gca   1981
Arg Met Gln Asp Lys Asn Pro Tyr Ser Phe Gln Ser Asp Val Tyr Ala
625                 630                 635                 640

ttt ggg att gtt ctg tat gaa ttg atg act gga cag tta cct tat tca   2029
Phe Gly Ile Val Leu Tyr Glu Leu Met Thr Gly Gln Leu Pro Tyr Ser
                645                 650                 655

aac atc aac aac agg gac cag ata att ttt atg gtg gga cga gga tac   2077
Asn Ile Asn Asn Arg Asp Gln Ile Ile Phe Met Val Gly Arg Gly Tyr
            660                 665                 670

ctg tct cca gat ctc agt aag gta cgg agt aac tgt cca aaa gcc atg   2125
Leu Ser Pro Asp Leu Ser Lys Val Arg Ser Asn Cys Pro Lys Ala Met
        675                 680                 685

aag aga tta atg gca gag tgc ctc aaa aag aaa aga gat gag aga cca   2173
Lys Arg Leu Met Ala Glu Cys Leu Lys Lys Lys Arg Asp Glu Arg Pro
    690                 695                 700

ctc ttt ccc caa att ctc gcc tct att gag ctg ctg gcc cgc tca ttg   2221
Leu Phe Pro Gln Ile Leu Ala Ser Ile Glu Leu Leu Ala Arg Ser Leu
705                 710                 715                 720
```

```
cca aaa att cac cgc agt gca tca gaa ccc tcc ttg aat cgg gct ggt   2269
Pro Lys Ile His Arg Ser Ala Ser Glu Pro Ser Leu Asn Arg Ala Gly
                725                 730                 735

ttc caa aca gag gat ttt agt cta tat gct tgt gct tct cca aaa aca   2317
Phe Gln Thr Glu Asp Phe Ser Leu Tyr Ala Cys Ala Ser Pro Lys Thr
            740                 745                 750

ccc atc cag gca ggg gga tat ggt gcg ttt cct gtc cac tga           2359
Pro Ile Gln Ala Gly Gly Tyr Gly Ala Phe Pro Val His
        755                 760                 765

aacaaatgag tgagagagtt caggagagta gcaacaaaag gaaaataaat gaacatatgt 2419

ttgcttatat gttaaattga ataaaatact ctctttttt ttaaggtgga aaaaaaaaaa 2479

aaaaaaaaaa aaaaaaaaaa aaaaaaaacc c                                2510


<210> 4
<211> 765
<212> PRT
<213> Homo sapiens

<400> 4
Met Ala Ala Leu Ser Gly Gly Gly Gly Gly Gly Ala Glu Pro Gly Gln
  1               5                  10                  15
Ala Leu Phe Asn Gly Asp Met Glu Pro Glu Ala Gly Ala Gly Arg Pro
             20                  25                  30
Ala Ala Ser Ser Ala Ala Asp Pro Ala Ile Pro Glu Glu Val Trp Asn
         35                  40                  45
Ile Lys Gln Met Ile Lys Leu Thr Gln Glu His Ile Glu Ala Leu Leu
     50                  55                  60
Asp Lys Phe Gly Gly Glu His Asn Pro Pro Ser Ile Tyr Leu Glu Ala
 65                  70                  75                  80
Tyr Glu Glu Tyr Thr Ser Lys Leu Asp Ala Leu Gln Gln Arg Glu Gln
                 85                  90                  95
Gln Leu Leu Glu Ser Leu Gly Asn Gly Thr Asp Phe Ser Val Ser Ser
            100                 105                 110
Ser Ala Ser Met Asp Thr Val Thr Ser Ser Ser Ser Ser Ser Leu Ser
        115                 120                 125
Val Leu Pro Ser Ser Leu Ser Val Phe Gln Asn Pro Thr Asp Val Ala
    130                 135                 140
Arg Ser Asn Pro Lys Ser Pro Gln Lys Pro Ile Val Arg Val Phe Leu
145                 150                 155                 160
Pro Asn Lys Gln Arg Thr Val Val Pro Ala Arg Cys Gly Val Thr Val
                165                 170                 175
Arg Asp Ser Leu Lys Lys Ala Leu Met Met Arg Gly Leu Ile Pro Glu
            180                 185                 190
Cys Cys Ala Val Tyr Arg Ile Gln Asp Gly Glu Lys Lys Pro Ile Gly
        195                 200                 205
Trp Asp Thr Asp Ile Ser Trp Leu Thr Gly Glu Glu Leu His Val Glu
    210                 215                 220
Val Leu Glu Asn Val Pro Leu Thr Thr His Asn Phe Val Arg Lys Thr
225                 230                 235                 240
Phe Phe Thr Leu Ala Phe Cys Asp Phe Cys Arg Lys Leu Leu Phe Gln
                245                 250                 255
Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Gln Arg Cys Ser
            260                 265                 270
```

```
Thr Glu Val Pro Leu Met Cys Val Asn Tyr Asp Gln Leu Asp Leu Leu
        275                 280                 285
Phe Val Ser Lys Phe Phe Glu His His Pro Ile Pro Gln Glu Glu Ala
    290                 295                 300
Ser Leu Ala Glu Thr Ala Leu Thr Ser Gly Ser Ser Pro Ser Ala Pro
305                 310                 315                 320
Ala Ser Asp Ser Ile Gly Pro Gln Ile Leu Thr Ser Pro Ser Pro Ser
                325                 330                 335
Lys Ser Ile Pro Ile Pro Gln Pro Phe Arg Pro Ala Asp Glu Asp His
            340                 345                 350
Arg Asn Gln Phe Gly Gln Arg Asp Arg Ser Ser Ser Ala Pro Asn Val
        355                 360                 365
His Ile Asn Thr Ile Glu Pro Val Asn Ile Asp Asp Leu Ile Arg Asp
    370                 375                 380
Gln Gly Phe Arg Gly Asp Gly Gly Ser Thr Thr Gly Leu Ser Ala Thr
385                 390                 395                 400
Pro Pro Ala Ser Leu Pro Gly Ser Leu Thr Asn Val Lys Ala Leu Gln
                405                 410                 415
Lys Ser Pro Gly Pro Gln Arg Glu Arg Lys Ser Ser Ser Ser Ser Glu
            420                 425                 430
Asp Arg Asn Arg Met Lys Thr Leu Gly Arg Arg Asp Ser Ser Asp Asp
        435                 440                 445
Trp Glu Ile Pro Asp Gly Gln Ile Thr Val Gly Gln Arg Ile Gly Ser
    450                 455                 460
Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp His Gly Asp Val Ala
465                 470                 475                 480
Val Lys Met Leu Asn Val Thr Ala Pro Thr Pro Gln Gln Leu Gln Ala
                485                 490                 495
Phe Lys Asn Glu Val Gly Val Leu Arg Lys Thr Arg His Val Asn Ile
            500                 505                 510
Leu Leu Phe Met Gly Tyr Ser Thr Lys Pro Gln Leu Ala Ile Val Thr
        515                 520                 525
Gln Trp Cys Glu Gly Ser Ser Leu Tyr His His Leu His Ile Ile Glu
    530                 535                 540
Thr Lys Phe Glu Met Ile Lys Leu Ile Asp Ile Ala Arg Gln Thr Ala
545                 550                 555                 560
Gln Gly Met Asp Tyr Leu His Ala Lys Ser Ile Ile His Arg Asp Leu
                565                 570                 575
Lys Ser Asn Asn Ile Phe Leu His Glu Asp Leu Thr Val Lys Ile Gly
            580                 585                 590
Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp Ser Gly Ser His Gln
        595                 600                 605
Phe Glu Gln Leu Ser Gly Ser Ile Leu Trp Met Ala Pro Glu Val Ile
    610                 615                 620
Arg Met Gln Asp Lys Asn Pro Tyr Ser Phe Gln Ser Asp Val Tyr Ala
625                 630                 635                 640
Phe Gly Ile Val Leu Tyr Glu Leu Met Thr Gly Gln Leu Pro Tyr Ser
                645                 650                 655
Asn Ile Asn Asn Arg Asp Gln Ile Ile Phe Met Val Gly Arg Gly Tyr
            660                 665                 670
Leu Ser Pro Asp Leu Ser Lys Val Arg Ser Asn Cys Pro Lys Ala Met
        675                 680                 685
Lys Arg Leu Met Ala Glu Cys Leu Lys Lys Lys Arg Asp Glu Arg Pro
    690                 695                 700
Leu Phe Pro Gln Ile Leu Ala Ser Ile Glu Leu Leu Ala Arg Ser Leu
705                 710                 715                 720
Pro Lys Ile His Arg Ser Ala Ser Glu Pro Ser Leu Asn Arg Ala Gly
                725                 730                 735
Phe Gln Thr Glu Asp Phe Ser Leu Tyr Ala Cys Ala Ser Pro Lys Thr
            740                 745                 750
```

```
Pro Ile Gln Ala Gly Gly Tyr Gly Ala Phe Pro Val His
        755                 760                 765



<210> 5
<211> 2977
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (130)..(2076)

<400> 5
ccgaatgtga ccgcctcccg ctccctcacc cgccgcgggg aggaggagcg ggcgagaagc 60

tgccgccgaa cgacaggacg ttggggcggc ctggctccct caggtttaag aattgtttaa 120

gctgcatca atg gag cac ata cag gga gct tgg aag acg atc agc aat ggt 171
          Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly
            1               5                  10

ttt gga ttc aaa gat gcc gtg ttt gat ggc tcc agc tgc atc tct cct   219
Phe Gly Phe Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro
 15                  20                  25                  30

aca ata gtt cag cag ttt ggc tat cag cgc cgg gca tca gat gat ggc   267
Thr Ile Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly
                35                  40                  45

aaa ctc aca gat cct tct aag aca agc aac act atc cgt gtt ttc ttg   315
Lys Leu Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu
            50                  55                  60

ccg aac aag caa aga aca gtg gtc aat gtg cga aat gga atg agc ttg   363
Pro Asn Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu
        65                  70                  75

cat gac tgc ctt atg aaa gca ctc aag gtg agg ggc ctg caa cca gag   411
His Asp Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu
    80                  85                  90

tgc tgt gca gtg ttc aga ctt ctc cac gaa cac aaa ggt aaa aaa gca   459
Cys Cys Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala
 95                  100                 105                 110

cgc tta gat tgg aat act gat gct gcg tct ttg att gga gaa gaa ctt   507
Arg Leu Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu
                115                 120                 125

caa gta gat ttc ctg gat cat gtt ccc ctc aca aca cac aac ttt gct   555
Gln Val Asp Phe Leu Asp His Val Pro Leu Thr Thr His Asn Phe Ala
            130                 135                 140

cgg aag acg ttc ctg aag ctt gcc ttc tgt gac atc tgt cag aaa ttc   603
Arg Lys Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe
        145                 150                 155

ctg ctc aat gga ttt cga tgt cag act tgt ggc tac aaa ttt cat gag   651
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```
Leu Leu Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu
    160                 165                 170

cac tgt agc acc aaa gta cct act atg tgt gtg gac tgg agt aac atc   699
His Cys Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile
175                 180                 185                 190

aga caa ctc tta ttg ttt cca aat tcc act att ggt gat agt gga gtc   747
Arg Gln Leu Leu Leu Phe Pro Asn Ser Thr Ile Gly Asp Ser Gly Val
                195                 200                 205

cca gca cta cct tct ttg act atg cgt cgt atg cga gag tct gtt tcc   795
Pro Ala Leu Pro Ser Leu Thr Met Arg Arg Met Arg Glu Ser Val Ser
            210                 215                 220

agg atg cct gtt agt tct cag cac aga tat tct aca cct cac gcc ttc   843
Arg Met Pro Val Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe
        225                 230                 235

acc ttt aac acc tcc agt ccc tca tct gaa ggt tcc ctc tcc cag agg   891
Thr Phe Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg
    240                 245                 250

cag agg tcg aca tcc aca cct aat gtc cac atg gtc agc acc acg ctg   939
Gln Arg Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu
255                 260                 265                 270

cct gtg gac agc agg atg att gag gat gca att cga agt cac agc gaa   987
Pro Val Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu
                275                 280                 285

tca gcc tca cct tca gcc ctg tcc agt agc ccc aac aat ctg agc cca  1035
Ser Ala Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro
            290                 295                 300

aca ggc tgg tca cag ccg aaa acc ccc gtg cca gca caa aga gag cgg  1083
Thr Gly Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg
        305                 310                 315

gca cca gta tct ggg acc cag gag aaa aac aaa att agg cct cgt gga  1131
Ala Pro Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly
    320                 325                 330

cag aga gat tca agc tat tat tgg gaa ata gaa gcc agt gaa gtg atg  1179
Gln Arg Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met
335                 340                 345                 350

ctg tcc act cgg att ggg tca ggc tct ttt gga act gtt tat aag ggt  1227
Leu Ser Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly
                355                 360                 365

aaa tgg cac gga gat gtt gca gta aag atc cta aag gtt gtc gac cca  1275
Lys Trp His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro
            370                 375                 380

acc cca gag caa ttc cag gcc ttc agg aat gag gtg gct gtt ctg cgc  1323
Thr Pro Glu Gln Phe Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg
        385                 390                 395

aaa aca cgg cat gtg aac att ctg ctt ttc atg ggg tac atg aca aag  1371
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Lys Thr Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys
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gac aac ctg gca att gtg acc cag tgg tgc gag ggc agc agc ctc tac   1419
Asp Asn Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr
415                 420                 425                 430

aaa cac ctg cat gtc cag gag acc aag ttt cag atg ttc cag cta att   1467
Lys His Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile
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gac att gcc cgg cag acg gct cag gga atg gac tat ttg cat gca aag   1515
Asp Ile Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys
            450                 455                 460

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Asn Ile Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu
        465                 470                 475

ggc tta aca gtg aaa att gga gat ttt ggt ttg gca aca gta aag tca   1611
Gly Leu Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser
    480                 485                 490

cgc tgg agt ggt tct cag cag gtt gaa caa cct act ggc tct gtc ctc   1659
Arg Trp Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu
495                 500                 505                 510

tgg atg gcc cca gag gtg atc cga atg cag gat aac aac cca ttc agt   1707
Trp Met Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser
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Phe Gln Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met
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acg ggg gag ctt cct tat tct cac atc aac aac cga gat cag atc atc   1803
Thr Gly Glu Leu Pro Tyr Ser His Ile Asn Asn Arg Asp Gln Ile Ile
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ttc atg gtg ggc cga gga tat gcc tcc cca gat ctt agt aag cta tat   1851
Phe Met Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Lys Leu Tyr
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aag aac tgc ccc aaa gca atg aag agg ctg gta gct gac tgt gtg aag   1899
Lys Asn Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys
575                 580                 585                 590

aaa gta aag gaa gag agg cct ctt ttt ccc cag atc ctg tct tcc att   1947
Lys Val Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile
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gag ctg ctc caa cac tct cta ccg aag atc aac cgg agc gct tcc gag   1995
Glu Leu Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu
            610                 615                 620

cca tcc ttg cat cgg gca gcc cac act gag gat atc aat gct tgc acg   2043
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ctg acc acg tcc ccg agg ctg cct gtc ttc tag ttgactttgc acctgtcttc 2096
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Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
        35                  40                  45
Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50                  55                  60
Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65                  70                  75                  80
Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                85                  90                  95
Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala Arg Leu
            100                 105                 110
Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
        115                 120                 125
Asp Phe Leu Asp His Val Pro Leu Thr Thr His Asn Phe Ala Arg Lys
    130                 135                 140
Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145                 150                 155                 160
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                165                 170                 175
Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
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Leu Leu Leu Phe Pro Asn Ser Thr Ile Gly Asp Ser Gly Val Pro Ala
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Leu Pro Ser Leu Thr Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210                 215                 220
Pro Val Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225                 230                 235                 240
Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
                245                 250                 255
Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu Pro Val
            260                 265                 270
Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275                 280                 285
Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
    290                 295                 300
Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
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Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
                325                 330                 335
Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340                 345                 350
Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355                 360                 365
His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
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385                 390                 395                 400
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Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465                 470                 475                 480
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    530                 535                 540
Glu Leu Pro Tyr Ser His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545                 550                 555                 560
Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Lys Leu Tyr Lys Asn
                565                 570                 575
Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580                 585                 590
Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
        595                 600                 605
Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: artificial primer

<400> 9
agtgccagcg gggctgctaa a 21

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(74) Agent: **VOSSIUS & PARTNER**; Siebertstrasse 4, 81675 München (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
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(88) Date of publication of the international search report:
2 October 2003

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WO 02/026246 A3

(54) Title: PHARMACEUTICAL COMPOSITIONS COMPRISING POLYNUCLEOTIDES ENCODING A RAF PROTEIN

(57) Abstract: Disclosed are pharmaceutical compositions comprising polynucleotides encoding a Raf protein, vectors, host cells, polypeptides encoded by said polynucleotides as well as agonists or antagonists thereof. Furthermore, described are uses of such pharmaceutical compositions for preventing or treating pathological conditions in which endothelial cells are involved or affected. Finally, methods for screening compounds acting as agonists or antagonists as well as diagnostic compositions and methods are disclosed.

INTERNATIONAL SEARCH REPORT

Internatl Application No
PCT/EP 01/11282

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/45

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, CHEM ABS Data, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 114 517 A (MONIA BRETT P ET AL) 5 September 2000 (2000-09-05) column 1, line 7 - line 53; claims 1-4 column 2, line 24 -column 3, line 4 column 4, line 3 - line 16 column 4, line 39 -column 5, line 17 column 7, line 26 - line 54 column 17, line 1 - line 16 --- -/--	1,6-14, 20,21, 23,24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 February 2003	05/03/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Charles, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internatk Application No
PCT/EP 01/11282

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ERHARDT P ET AL: "B-Raf inhibits programmed cell death downstream of cytochrome c release from mitochondria by activating the MEK/Erk pathway." MOLECULAR AND CELLULAR BIOLOGY. UNITED STATES AUG 1999, vol. 19, no. 8, August 1999 (1999-08), pages 5308-5315, XP002231909 ISSN: 0270-7306 page 5308, right-hand column, paragraph 3 page 5312, right-hand column, paragraph 1 -page 5313, right-hand column, paragraph 1 ---	1,2
X	US 5 618 670 A (RAPP ULF R ET AL) 8 April 1997 (1997-04-08) column 1, line 13 - line 17; claim 1 column 2, line 63 -column 3, line 40 column 5, line 13 -column 6, line 14 ---	23,24
X,P	WO 01 12210 A (SCRIPPS RESEARCH INST ;HOOD JOHN (US); CHERESH DAVID A (US); ELICE) 22 February 2001 (2001-02-22) page 1, line 8 - line 25; claims 1-3,19-21,64-67 page 3, line 2 -page 5, line 20 ---	1,6-19
E	EP 1 176 200 A (SWITCH BIOTECH AG) 30 January 2002 (2002-01-30) page 3, line 17 - line 47; claims 2,3,8-10 page 4, line 12 - line 46 ---	1,2,6-8, 20,21, 23,24
E	WO 01 96535 A (SCHEBO BIOTECH AG ;RAPP ULF R (DE); EIGENBRODT ERICH (DE)) 20 December 2001 (2001-12-20) page 1, line 8 - line 15; claims 1-3,5-7,11-13 page 6, line 7 -page 12, line 10 ---	1,6,8, 12,13, 20,21, 23,24
A	WO 97 36587 A (MERCK & CO INC ;HEIMBROOK DAVID C (US); OLIFF ALLEN I (US); STIRDI) 9 October 1997 (1997-10-09) page 1, line 5 - line 31; claims 1-5 page 4, line 25 -page 13, line 23 ---	1,6,8, 12,13
A	SITHANANDAM G ET AL: "B-RAF AND A B-RAF PSEUDOGENE ARE LOCATED ON 7Q IN MAN" ONCOGENE, BASINGSTOKE, HANTS, GB, vol. 7, no. 4, April 1992 (1992-04), pages 795-799, XP008013456 ISSN: 0950-9232 page 798, left-hand column, line 1 ---	1,6,8, 12,13

-/--

INTERNATIONAL SEARCH REPORT

Interna Application No
PCT/EP 01/11282

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	XU X S ET AL: "A role for c- Raf kinase and Ha-Ras in cytokine-mediated induction of cell adhesion molecules." JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 DEC 11) 273 (50) 33230-8. , XP002231910 page 33231, right-hand column, paragraph 1 page 33238, left-hand column, paragraph 2 - paragraph 3 ---	1,6
A	WO 93 04170 A (US GOVERNMENT) 4 March 1993 (1993-03-04) page 4, line 4 - line 16; claims 1-8 -----	1

INTERNATIONAL SEARCH REPORT

Inte ıal application No.
PCT/EP 01/11282

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna pplication No
PCT/EP 01/11282

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6114517 A	05-09-2000	AU 1842900 A	26-06-2000
		EP 1137658 A1	04-10-2001
		JP 2002531574 T	24-09-2002
		WO 0034303 A1	15-06-2000
US 5618670 A	08-04-1997	US 5156841 A	20-10-1992
		US 5869308 A	09-02-1999
		AT 178357 T	15-04-1999
		AU 666336 B2	08-02-1996
		AU 2658992 A	27-04-1993
		CA 2118724 A1	01-04-1993
		DE 69228824 D1	06-05-1999
		DE 69228824 T2	09-12-1999
		EP 0612353 A1	31-08-1994
		JP 8506000 T	02-07-1996
		WO 9306248 A1	01-04-1993
WO 0112210 A	22-02-2001	AU 6763300 A	13-03-2001
		CN 1378457 T	06-11-2002
		CZ 20020449 A3	14-08-2002
		EP 1210099 A1	05-06-2002
		NO 20020718 A	10-04-2002
		SK 2142002 A3	02-07-2002
		WO 0112210 A1	22-02-2001
EP 1176200 A	30-01-2002	DE 10030149 A1	10-01-2002
		AU 5199401 A	01-08-2002
		EP 1114862 A2	11-07-2001
		EP 1176200 A2	30-01-2002
		JP 2001292783 A	23-10-2001
		JP 2002356436 A	13-12-2002
		US 2002086019 A1	04-07-2002
WO 0196535 A	20-12-2001	DE 10029131 A1	03-01-2002
		AU 7234301 A	24-12-2001
		WO 0196535 A2	20-12-2001
WO 9736587 A	09-10-1997	AU 727939 B2	04-01-2001
		AU 2722197 A	22-10-1997
		CA 2250232 A1	09-10-1997
		EP 0906099 A1	07-04-1999
		JP 2000504023 T	04-04-2000
		WO 9736587 A1	09-10-1997
WO 9304170 A	04-03-1993	AU 2552492 A	16-03-1993
		WO 9304170 A1	04-03-1993

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